5 Medical Lib.

Vol. XVII APRIL, 1932 APR 2 6 1932 No. 7

The Journal of Laboratory and Clinical Medicine

WARREN T. VAUGHAN, M.D., EDITOR
808 Professional Building
Richmond, Va.

ASSOCIATE EDITORS

Pharmacology
DENNIS E. JACKSON, M.D.
University of Cincinnati, Cincinnati

Pathology
PAUL G. WOOLLEY, M.D.
Pasadena

Physiology
J. J. R. MACLEOD, M.B.
Physiological Laboratory, Marischal College, Aberdeen, Scotland

Tuberculosis
GERALD B. WEBB, M.D.
Cragmor Sanatorium, Colorado Springs

Clinical Pathology
W. C. MacCARTY, M.D.
Mayo Clinic, Rochester, Minn.

T. B. MAGATH, M.D.
Mayo Clinic, Rochester, Minn.

Biochemistry
VICTOR C. MYERS, Ph.D.
Western Reserve University, Cleveland

Experimental Medicine
RUSSELL L. HADEN, M.D.
Cleveland Clinic, Cleveland

Immunology
JOHN A. KOLMER, M.D.
University of Pennsylvania, Philadelphia

ROBERT A. KILDUFFE, M.D.
Atlantic City, N. J.

Internal Medicine
GEORGE HERRMANN, M.D.
University of Texas, Galveston

Bacteriology
M. H. SOULE, Sc.D.
University of Michigan, Ann Arbor

Surgery
DEAN LEWIS, M.D.
Johns Hopkins University, Baltimore

PUBLISHED BY THE C. V. MOSBY COMPANY, 3523-25 PINE BLVD., ST. LOUIS.

Entered at the Post Office at St. Louis, Mo., as Second-Class Matter. Additional Entry as Second-Class Matter at Fulton, Mo.

CAPACITY TO PAY . . .

Membership limited to ethical Physicians, Surgeons and Dentists

IN these days when we hear so much of the suggestion that the liability under any treaty, contract or agreement should be determined by "the capacity to pay," it is important that any insurance company should have sufficient sound assets to enable it to pay all obligations in full. It is with a feeling of pride and satisfaction that we present the report showing unencumbered assets exceeding $1,000,000.00 at the close of thirty years' continuous operation, under the same management.

In 1931 we paid for sick and accident claims over $528,000.00, saved and invested for future protection over $56,000.00, making over $584,000.00 used for benefits out of the $638,000.00 collected from the members (over 91%).

Physicians Casualty Association
E. E. Elliott, Secretary-Treasurer

Physicians Health Association
Omaha, Nebraska

Importance of Limited Diets

Often Causing Serious Secondary Effects

In the Jour. A.M.A., Nov. 14, 1931, Dr. Russell M. Wilder, Chicago, says:

> "I should like to mention two cases of pellagra that developed in patients that were under a dietary treatment for epilepsy.
> "In the two cases referred to—the addition of Brewers' Yeast alone, without other alteration of the diet, promptly resulted in a cure. Since then I have made a practice of including yeast in all my epileptic diets and thus far have not again encountered this complication."

Likewise, the "restricted diets" of diabetes, obesity and the like, often reduce the content of vitamine-B (F & G) to a dangerously low level.

Dr. H. C. Sherman (Jour. A.M.A., Nov. 14, 1931) says:

> "—so a liberal intake of vitamine-G contributes to a better than average nutritional condition and thus to what McCollum and Simmonds have aptly termed 'the preservation of the characteristics of youth.'"

BREWERS' YEAST-HARRIS [Dry Powder]

has been adopted by the U. S. Public Health Service for the successful treatment of pellagra of the south. It is indicated in the following conditions:

Accessory to deficient diets
Anemias
Convalescence
Immunity Building

Yeast Vitamine-Harris Tablets are made from a concentrate of this same yeast. They are smaller in volume, palatable, contain a known amount of vitamine-B, and are used by physicians where smaller dosage is required.

Samples sent on request by

The Harris Laboratories, Inc.

Tuckahoe, New York

The Journal of Laboratory and Clinical Medicine

VOL. XVII ST. LOUIS, MO., APRIL, 1932 NO. 7

SYMPOSIUM ON CLINICAL BACTERIOLOGY

(Concluded from March issue)

SPUTUM EXAMINATION IN PULMONARY TUBERCULOSIS*

BY MAX PINNER, M.D., TUCSON, ARIZONA

THE topic indicated in the title of this paper seems hardly worthy for a journal article. Such elementary discussion should really be confined to textbooks. But in spite of the increased emphasis put on tuberculosis teaching, the following quotation is still a fair statement of facts. This may justify the subsequent pages.

"A plea for the general tightening up of the technic of collection and examination of the sputum may not be out of place here. The character of many specimens received in laboratories and their slipshod handling afterward are enough to make one wonder as to just how trustworthy the average of reports may be. I have seen senior house-officers in first-class hospitals begin with the sputum in a narrow-neck dark bottle, then introduce the platinum loop blindly, withdraw whatever mucus clung by chance to the loop, smear this on a slide, stain, and, after a rather casual survey—search would be altogether too inaccurate a term—report the specimen as negative. In other words, there were violations of good technic at every single stage of the examination." (Krause.[30])

1. COLLECTION OF SPUTUM

The first and a very important step in the examination of sputum for tubercle bacilli is the proper collection of sputum. It should not be necessary to mention the need for bacteriologic cleanliness of the containers, but since sterilization does not necessarily destroy the morphologic entity of tubercle bacilli, it is preferable to use containers for sputum specimens only once, such as paraffinized cardboard boxes or ice cream boxes.

From manifold experience it seems imperative to emphasize the necessity of properly instructing the patient. Too many times the patient's assurance

*From the Laboratories of the Desert Sanatorium and Institute of Research.

that he does not expectorate is accepted with facile acquiescence, although the patient—frequently hardly initiated in the customs and parlance of things medical—is wholly ignorant of the meaning and the clinical significance of such terms as "sputum" and "expectoration." A simple and patient instruction—if need be in the vernacular rather than in medical terms—will often be productive of ample specimens the sources of which the patient had been quite unaware. The patient must and will learn to differentiate between true sputum and nasopharyngeal drippings and saliva, and he can be educated to avoid sending along with his sputum such foreign bodies as are of no medical interest. In many instances it is necessary to have sputum collected over a number of days.

In young children, it is usually impossible to obtain sputum. Tubercle bacilli may, however, be found in smears from pharynx, tonsils, or larynx, in feces, and in gastric contents. The gastric contents, preferably collected in the morning before breakfast, by gastric lavage, are treated by concentration methods exactly in the same manner as sputum. (Armand Delille and Vibert.[2])

The methods for demonstrating tubercle bacilli in feces are essentially the same as those for sputum, discussed later. Animal inoculation should be used whenever direct smears show no bacilli, since cultural methods are somewhat less successful with stools than with sputum.

2. PREPARATION OF DIRECT SMEARS

In every complete sputum report, the gross appearance of the specimen should be mentioned. Although there is no sputum characteristic in its gross appearance for tuberculosis, the nature of a specimen may frequently form an important link in the clinical evidence, diagnostically or prognostically. Any special gross feature of the sputum should be carefully reported: layer formation, especially in association with basal lesions, is of great diagnostic significance. The relative amount of pus, as seen macroscopically, must be noted. Much pus in the absence of tubercle bacilli throws the weight against pulmonary tuberculosis; here again correlation with clinical and roentgenologic findings goes a long way toward establishing a diagnosis. When the diagnosis of pulmonary tuberculosis is established, fluctuations in the relative amount of pus are prognostically significant. And may it be emphasized here, that in pulmonary tuberculosis one need not search for the socalled pyogenic organisms to explain the presence of pus in the sputum; the tubercle bacillus is itself a pyogenic organism. Socalled "secondary infection" plays undoubtedly an insignificant rôle. A true secondary infection of the tissue is exceedingly rare, while secondary infection of the *necrotic contents* of cavities is common, but apparently without clinical significance. While emphasizing the often neglected need of reporting the gross appearance of sputum, it should be remembered that no description can substitute for the visual impression that the clinician should insist upon procuring for himself at frequent intervals. For the laboratory it must be emphasized again and again, that any kind of sputum may contain tubercle bacilli, be it ever so small in amount, or be it ever so "innocent-looking," watery clear, like saliva. Therefore: no specimen may ever be discharged without careful bacteriologic study. Occasionally patients with the clinical evidence of pulmonary tuberculosis are seen who produce practically no sputum,

but who have an occasional hemorrhage. In such cases the expectorated blood should be examined for bacilli; the search will be successful in a number of instances. The amount of twenty-four-hour specimens should be reported by weight and not by volume.

It is a good rule to start every sputum examination with a study of a fresh, unstained smear. This may not be feasible where a large volume of routine work has to be done. Since the results of fresh smear studies are chiefly of importance in nontuberculous pulmonary disease, these findings will not be discussed here, with the exception of the demonstration of elastic fibers. Although special methods for their demonstration are advocated, they can easily be found in the fresh unstained smear. The presence of elastic fibers indicates destruction of pulmonary tissue, regardless of the etiologic agent. If they are present in sputum in which careful, complete, and repeated studies fail to demonstrate tubercle bacilli the probability is very great that tuberculosis is not the cause of the destructive pulmonary lesion.

In preparing smears for the detection of tubercle bacilli three points, frequently neglected, are particularly important:

1. Select with utmost care a particle of sputum.
2. Make thin and even smears.
3. Make a very light counterstain.

Point 1: If this step is performed with care and understanding, a great deal of time will be saved. Since bacilli are not evenly distributed, but are massed together in purulent or cheesy particles, such particles must be selected for examination. If the specimen is spread out in a Petri dish and viewed against a black background, the proper selection of particles becomes an easy matter.

Point 2: Unless this precaution is taken, decolorization is difficult and bacilli may remain hidden under cellular or mucoid material.

Point 3: It is my experience that this rule is most frequently violated, not only by pseudotechnicians, but by expert workers, too, and by competent textbooks. Loeffler's methylene blue, diluted with water 1:80, and applied for five to ten seconds gives a sufficiently distinct counterstain without obscuring tubercle bacilli.

A brief discussion of stains for tubercle bacilli follows:

Bacterial Stains.—Most methods use basic fuchsin. Herman[18, 19] advocated crystal violet, and his method is indeed very satisfactory for tissue stains, but it has no apparent advantages over the time-honored fuchsin stains for sputum smears.

Mordants.—The most commonly used mordant is carbolic acid. In Herman's method, ammonium carbonate is substituted.

Decolorizing Agents.—The most satisfactory decolorizing agent is a 3 per cent solution of concentrated hydrochloric acid in 95 per cent alcohol. Nitric acid which is advocated in a number of methods is less satisfactory, because traces of nitrous acid may decolorize tubercle bacilli (Krause, quoted by Willis),[56] Weichselbaum, in a method modified by Gabbet,[17] used decolorization and counterstaining agent in one solution. This procedure, although somewhat speedier,

cannot be recommended because the process of decolorization cannot be controlled properly. Konrich[28] reduced the fuchsin to its colorless leucobase by the use of sodium sulphite. This method has advantages when, for some reasons, alcohol is difficult to obtain, but it is somewhat more cumbersome since the sodium sulphite solution must always be freshly prepared.

Counterstain.—Methylene blue, as used in the Ziehl-Neelsen method is quite satisfactory because it produces sharp definition and good contrast. It should, as any other counterstain, be used in such a dilution, as to stain the cells only a faint blue. If the counterstain is applied too heavily, tubercle bacilli may be obscured by it; and even if this be not the case, the bacilli are much less readily seen. Spengler[48] advised picric acid as counterstain. Since, however, picric acid produces only a very faint yellowish background without definitely outlining the cellular elements, the eye fatigues much more quickly examining slides stained by this method. A further disadvantage of this method is the fact that it does not enable the observer to recognize cellular elements and other bacteria. Brilliant green, in proper dilution, is as good a counterstain as methylene blue.

The literature on stains for tubercle bacilli is immense; new methods and modifications have been advocated ad infinitum, but it is a highly significant fact that whenever comparative studies are made, the Ziehl-Neelsen method, with or without minor modifications, remains victorious as an accurate and simple method. (See for example Corper, 1926.) Inquiring in many laboratories, one finds that the vast majority of them uses the Ziehl-Neelsen method, although many have, as has the author, given fair trials to other methods. A recent modification seems to have slight though definite advantages in our experience, without complicating the procedure. This is Cooper's modification which yields excellently defined preparations.

The original Ziehl-Neelsen method, with rather insignificant deviations in the preparation of the staining solution, and Cooper's modification, follow:

I. *Ziehl-Neelsen*[37]

1. Saturated solution of basic fuchsin:

Basic fuchsin	2.0 c.c.
Alcohol, 95 per cent	100.0 c.c.

2. Carbolfuchsin solution:

Saturated solution of basic fuchsin	10.0 c.c.
Carbolic acid, aqueous 3 per cent	90.0 c.c.

Steam three minutes, wash, decolorize in 2.5 c.c. hydrochloric acid plus 97.5 c.c. 95 per cent alcohol. Wash, counterstain with Loeffler's methylene blue diluted with water 1: 80 for five to ten seconds, wash, dry.

II. *Ziehl-Neelsen (Cooper's modification)*[7]

Flood the slide with fresh carbolfuchsin to which 3 c.c. of 10 per cent sodium chloride per 100 c.c. is added; steam for four minutes and allow to cool until the precipitate forms. Wash with tap water. Decolorize with acid alcohol (5 c.c. nitric acid, sp. gr. 1.42 plus 95 c.c. of 95 per cent alcohol), wash, two minutes 95 per cent alcohol, wash, counterstain with 1 per cent brilliant green in 1: 10,000 sodium hydroxide for one minute, wash, dry. (For reasons previously mentioned, 2.5 - 3 per cent hydrochloric acid in 95 per cent alcohol is a more satisfactory decolorizing agent.)

Since Much's work on granula was published a number of staining methods have been elaborated with the purpose of staining both the classical acid-fast rods and Much's nonacid-fast granules. It is still very doubtful whether Much's

granules, the existence of which is not doubted, can be differentiated by these methods in sputum smears from other coccoid bodies. They can at the present time not be accepted as a diagnostic criterion. But since a considerable theoretical interest is attached to them, recently revived by the work of Sweany and of Kahn, it may be mentioned that the method of Weiss,[55] and the less cumbersome method of Kiefer, have yielded very satisfactory results in my hands.

Kiefer's Stain[26]

Carbolfuchsin	4 parts	by volume
Carbol methylviolet	3 parts	

If this mixture shows a metallic scum, add 95 per cent alcohol drop by drop until the scum disappears.

Fixing solution:

Iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	100 c.c.

Decolorizing solution:

Concentrated hydrochloric acid	10 c.c.
Alcohol, 95 per cent	50 c.c.
Acetone	40 c.c.

Counterstain:

1 per cent aqueous methylene blue.

Stain while heating ½ to ¾ minute, rinse; iodine solution 1 to 1½ minutes, rinse; decolorize ¾ to 1 minute, rinse; counterstain.

A number of staining methods have been devised to stain differentially both tubercle bacilli and elastic fibers in the same preparation. The two methods mentioned below fulfill this requirement fairly satisfactory, but it is doubtful whether they are full substitutes for a careful search for elastic fibers in the unstained preparation.

1. *Rappaport and Ellison.*[47]—Weigert's elastica stain three to five minutes, rinse, decolorize to a faint pink with 3 per cent hydrochloric acid in 95 per cent alcohol; rinse; counterstain with aqueous methylene blue.

2. Jessen[24] uses the Ziehl-Neelsen stain and counterstains for one or two minutes with:

Hematoxylin,	1.0 gm.
Lithium carbonate saturated solution,	1.0 c.c.
Absolute alcohol,	20.0 c.c.
Distilled water,	20.0 c.c.

Then rinse, cover for a few seconds with 28 per cent ferric chloride, rinse, dry.

In accordance with what was said in regard to the gross appearance of sputum, the microscopic reports should mention not only the presence or absence of tubercle bacilli, but also the type of cells seen (leucocytes, lymphocytes, epithelial cells), their relative number, and the presence of nonacid-fast microorganisms. This latter observation is clinically more significant than cultures for secondary microorganisms. It is obvious that a properly prepared smear will give a truer picture of the composition of the sputum flora than cultures in which primarily insignificant organisms may outgrow the truly predominant type.

3. CONCENTRATION METHODS

When examinations of direct smears fail to demonstrate tubercle bacilli, further studies should be made on the sputum, since rather large numbers of

bacilli may escape detection by the simple smear method. Corper has estimated that more than 100,000 bacilli must be present per c.c. of sputum for their detection on smear preparations. It should become a routine procedure to concentrate each suspected specimen (if negative by direct smears), to culture it, and inject it into a guinea pig if the preceding method has failed.

The first concentration method seems to have been reported by Biedert[3]; he boiled the sputum with a liberal amount of a 0.2 per cent solution of sodium hydroxide. Mühlhäuser,[39] and later Czaplewsky and Hensel[14] reported encouraging results with modifications of Biedert's original method.

Uhlenhuth and Xylander,[54] studying the bacteriolytic action of antiformin, a mixture of sodium hypochlorite and sodium hydrate, found that antiformin kills bacteria, but not tubercle bacilli, and that it destroys other organic material, such as cells. They recommended antiformin for the preparation of sputum or other contaminated material for the isolation of tubercle bacilli in pure culture. Soon, however, this procedure was found exceedingly useful for the bacterioscopic demonstration of tubercle bacilli.

The following procedure can be recommended: About 10 c.c. of sputum is mixed with a 20 per cent antiformin solution to secure an approximate final concentration of 10 per cent antiformin. For thin, watery sputa this concentration should be decreased; for heavy tenacious specimens, it should be increased. The sputum-antiformin mixture is incubated at 37° C. for about half an hour, during which time it must be shaken several times; or it may be kept in a shaking machine for an equal length of time. The antiformin will transform the sputum into a clear, homogeneous fluid, destroying and dissolving all formed elements, with the exception of tubercle bacilli. The time necessary for complete digestion varies with different specimens. If the preparation is made for the sole purpose of bacterioscopic examination, the period of contact between sputum and antiformin does not matter, as long as it is extended enough to effect a complete homogenization; but if one wishes to isolate tubercle bacilli in pure culture, the shortest period effecting complete clearing is the optimal one. After clearing is complete the mixture is centrifugalized at high speed (preferably after the addition of an equal amount of water—or alcohol if no cultural studies are intended—in order to reduce the specific gravity). The supernatant is discarded and the sediment is neutralized with hydrochloric acid. The sediment is then ready to be spread on slides and stained. Since the sediment washes off readily in staining, it should be fixed to the slide with a 10 to 20 per cent solution of egg albumen.

The most successful application of Biedert's principle, the homogenization of sputum by alkalis, is the procedure published by Petroff[44]; it has found wide acceptance. In Petroff's method the sputum is mixed with an equal amount of 4 per cent sodium hydroxide. Otherwise, the procedure is the same as with antiformin. Dilution before centrifugalization and fixation with egg albumen are unnecessary.

Sweany[52] has worked out a procedure, using the alkali digestion of sputum, which seems to be very useful for work on large scale.

4. CULTURAL METHODS FOR THE DEMONSTATION OF TUBERLE BACILLI

a. The Preparation of the Sputum.—In recent years cultural methods have been improved to a sufficient degree to play a significant rôle in the demonstration of tubercle bacilli in sputum. Two factors decide the success of cultural methods: (1) The preparation of the sputum in such a way that tubercle bacilli are concentrated within a relatively small amount of material without affecting

their vitality, and at the same time killing other microorganisms as completely as possible, and (2) suitable media.

The laborious washing method of Kitasato[29] which yielded very inconstant results is now of only historical interest. Uhlenhuth's antiformin and Petroff's sodium hydroxide methods were for a number of years the most successful procedures for concentrating tubercle bacilli without much impairment of their viability and for killing contaminating organisms. In recent years these methods have been rivaled, if not surpassed, by acid digestion as advocated by Löwenstein[34] and Sumoyoshi.[50]

Löwenstein's original procedure, however, cannot be recommended, because he used too high a concentration of acids. Hohn[21] treats the sputum with ten to twelve per cent sulphuric acid for twenty minutes, using 1 - 2 c.c. sputum and 10 c.c. sulphuric acid. After centrifugalization, the sediment, without neutralization is seeded on Lubenau's medium. Other authors recommended further reduction of the acid (Corper: 6 per cent), or the use of a 3 to 6 per cent hydrochloric acid, or (Corper[13]) 5 per cent oxalic acid. It has been our experience that we get somewhat better results with acids, and after extended trials we prefer to use a 3 volume per cent hydrochloric acid. For bacterioscopic work, the sodium hydroxide method is preferable, because it produces a smaller amount of sediment, and hence a more effective concentration of tubercle bacilli.

Petroff's sodium hydroxide method deserves preference over Uhlenhuth's antiformin method, because of its greater simplicity and because it causes probably a smaller percentage of mortality of tubercle bacilli. It is important to find the optimal time for digestion: not too short, because the contaminating organisms are not killed, not too long, because too many tubercle bacilli become sufficiently damaged to fail growing on artificial media. In accordance with Sweany and Evanoff,[53] I prefer using a 3 instead of a 4 per cent sodium hydroxide solution for cultural work. The technic is identical with that for the bacterioscopic concentration method previously mentioned; of course, throughout the whole procedure strict aseptic precautions must be maintained. The neutralized sediment is planted rather thinly on the slants; the tubes are then sealed with paraffine or sealing wax and a small needle puncture is made through the seal to provide a free supply of oxygen.

b. Culture Media for the Isolation of Tubercle Bacilli.—In the choice of culture media for the isolation of tubercle bacilli, it must be kept in mind that not all media on which tubercle bacilli in pure transplants grow well are suitable for isolation. Quite generally it can be said that liquid media are unsuitable, and that most agar media are unsuccessful; the same seems to be true of all so-called synthetic media.

The number of media advocated for isolating tubercle bacilli is so great that it is impossible to mention them all, especially since many are but modifications of previously tried and recommended media.

In the preparation of culture media, the following substances are used successfully: Serum, potatoes, glycerol, eggs, milk. Serum media were used by Koch in his first studies,[27] potato media were recommended by Pawlowsky[42] (1888) and found particularly suitable in connection with glycerol, the use of which was first recommended by Nocard and Roux[40] (1888). The British Royal Commis-

sion recommended that the potatoes be soaked in a sodium carbonate solution and Matzuschita[36] (1899) showed by comparative experiments that tubercle bacilli grow better on potatoes that are slightly alkalinized. Eggs, particularly egg yolk, were added to nutrient media by Capaldi[6] (1896) and highly recommended for the growing of tubercle bacilli, while media the chief component of which is egg were introduced by Dorset[15] (1902), before he knew of Capaldi's previous studies and by Lubenau[35] (1907). Matzuschita[36] substituted milk for broth in a gelatine-glycerol medium. Although, as will be evident from the brief historical review just presented, all recent culture media are but elaborations and modifications of principles well established by the turn of the century, the isolation of tubercle bacilli from contaminated material, has, due to the work of the last few years, advanced from a complicated and unreliable method, used almost exclusively for scientific study, to an easy and highly dependable technic in clinical-pathologic routine.

Some of the apparently most useful media are enumerated below. As far as possible, the original prescriptions are given. I would emphasize, however, that all the media which require inspissation, the several varieties of egg media, yield, in our experience, considerably better results when instead of the repeated inspissation as prescribed, they are kept in the Arnold sterilizer just long enough to cause coagulation. This brief sterilization yields only a very occasional contaminated tube. It is necessary, of course, to proceed as aseptically as possible in the preparation of the media. It should further be pointed out that the usual "overnight in the incubator" is a totally inadequate sterility test; media containing eggs, milk, or potatoes should be kept in the incubator for not less than three days.

1. *Lubenau's*[35] *Egg Medium* (1907).—Three parts of well-mixed whole eggs are added to one part of 5 per cent glycerol nutrient broth. This mixture is tubed and inspissated for two or three hours at 90° C.

Hohn[23] emphasized the necessity of adding a small amount of glycerol broth to each tube. He further recommended that some hemoglobin be added to the medium, in the following way: Sterile blood clots are pressed through a sterile wire screen; the drippings are centrifuged to remove the serum. The blood is then hemolyzed by the addition of an equal amount of sterile distilled water. This hemoglobin solution is added in the amount of 2 per cent to the medium before tubing. Quite recently, Hohn[23a] suggested further modifications; in this publication, his present procedure is reported in minute details.

2. *Petroff's*[44] *Gentian Violet Egg Medium* (1913).—A veal or beef infusion is prepared in the usual way, using 1000 gm. of meat with 1000 c.c. of a 15 per cent solution of glycerol in water. Eggs are mixed and filtered through gauze under sterile precautions, and one part of infusion is mixed with two parts of eggs. To each liter of this mixture is added 10 c.c. of a 1 per cent alcoholic solution of gentian violet. After mixing well, the medium is filtered through gauze, tubed, and inspissated as follows: First day at 85° C. until coagulated, second and third days at 75° C. for one hour.

3. *Sweany's*[53] *Veal-Egg-Glycerol Milk Medium* (1928).—This medium is prepared exactly like Petroff's medium, with the exception that the meat infusion is prepared with sterilized milk instead of with water, and that no dye is added. The milk is sterilized in live steam on two successive days for forty-five minutes.

Sweany recommends that sterilized cream, 10 per cent, be substituted for the glycerine in the above medium for the cultivation of bovine bacilli.

4. *Glycerol Potatoes (Corper's*[11] *Modification)* (1928).—The usual halved potato cylinders are soaked for one or two hours in a 1 per cent aqueous solution of anhydrous sodium carbonate containing 1: 75,000 crystal violet; then they are wiped off with a clean towel, put

in culture tubes to which 1.5 c.c. of a 5 per cent glycerol broth is added, and autoclaved at 15 pounds pressure for at least thirty minutes.

5. *Löwenstein's*[34a] *Modification of Egg Medium* (1931).—The following solution is prepared:

Monopotassium phosphate,	1.0 c.c.
Sodium citrate,	1.0 c.c.
Magnesium sulphate,	1.0 c.c.
Asparagin,	3.0 c.c.
Glycerol,	60.0 c.c.
Distilled water,	1000.00 c.c.

To each 150 c.c. of this solution are added 6 gm. potato flour and 12 c.c. glycerol. This mixture is boiled while stirring for fifteen minutes and kept at 56° C. for one hour. Then 4 eggs and 1 egg yolk are added and 5 c.c. of a 2 per cent Congo red or malachite green solution. After thorough mixing, the medium is filtered through sterile gauze, tubed, and inspissated on two subsequent days at 80 to 85° C. for two hours.

Löwenstein claims that peptone inhibits somewhat the growth of tubercle bacilli; and he devised, therefore, this peptone-free medium.

An egg yolk agar (a modification of Capaldi's medium) has recently been recommended by Herrold.[20] His first studies appear to be promising, but this medium has not as yet stood the practical test.

The choice of a concentration method and of a suitable medium is still much discussed. Each method, and each medium has its advocates, the most emphatic advocate being usually the originator of the particular procedure. A careful study of the literature leaves one undecided as to the respective merits of each procedure.

We have tried all methods and media mentioned in this review, and a good many more. We have used alkalis and acids in widely varying concentrations; we have made our own modifications. If we present here the procedure which we employ at the present time, it is not because we believe that it is better than any other one, or because we think it is final. It is because, so far, it has yielded the best results in our hands and because we can say, by well-controlled series of examinations, what this procedure will do. We should like to recommend it for further trial, and we hope that further experience will improve it.

Our Procedure for Isolating Tubercle Bacilli.—Approximately 10 c.c. of sputum and 10 c.c. of 3 per cent (by volume) of hydrochloric acid are thoroughly mixed in a sterile, stoppered 50 c.c. centrifuge-tube. The mixing is done by beating the material with a sterile glass rod or applicator. Then the tube is shaken frequently during a period of twenty minutes. The mixture is then centrifuged for ten minutes, thus leaving sputum and acid in contact for not more than thirty minutes. After decanting, the sediment is smeared in a thin layer over the surface of the slants.

The following two types of media are used, and a minimum of four tubes of each medium are seeded.

1. *A Modification of Sweany's Medium:*

Sterilized milk (see Sweany's medium),	200 c.c.
Beef infusion broth (not alkalinized),	200 c.c.
Whole eggs,	800 c.c.
Glycerol,	60 c.c.
Aqueous solution of malachite green (2 per cent),	60 c.c.

These ingredients are mixed, the well-beaten eggs being added last. The whole mixture is filtered through sterile gauze and tubed. The tubes, in slanting position, are coagulated

in the Arnold sterilizer for about half an hour; that is, just long enough to obtain the desired consistency.

2. *Petragnani's*[42] *Medium* (1926):

Milk,	900 c.c.
Potato flour,	36 gm.
Peptone,	6 gm.
Potato (egg size pieces),	6

This mixture is kept in a boiling water-bath with frequent stirring until it becomes sticky: after this it is left in the water-bath for from one to two hours. After cooling to 50° C., 24 whole eggs and 6 egg yolks, 70 c.c. of glycerol, and 60 c.c. of a 2 per cent aqueous solution of malachite green are added; the whole mixture is filtered through sterile gauze, tubed, and solidified in the same way as the preceding medium.

These media are kept in the incubator for not less than three days before use.

The majority of positive cultures will be obtained by the end of the second month; the earliest colonies may appear within ten days, but all negative tubes should be kept for no less than three months.

Colonies appearing on these media should always be examined in smear preparations; although I have invariably found that typical colonies are composed of typical acid-fast rods, I have been surprised to find repeatedly acid-fast rods in totally atypical colonies. Whether such apparently typical organisms with atypical colony formation are true tubercle bacilli is impossible to say at the present time. Some ten strains are being studied in regard to pathogenicity and tuberculin production. At all events it seems to be a good rule to examine all colonies, regardless of their appearance before discarding the slants as "contaminated." Several authors have observed that scrapings from the surface of apparently sterile slants contain occasionally acid-fast rods; here again it is still undecided whether some or all or none of them are true tubercle bacilli.

c. The Efficiency of Cultural Methods.—It has been claimed by several writers that culture methods (usually a specific culture method) is fully as reliable as guinea pig inoculation. It is obvious, however, that so far no convincing proof has been adduced to substantiate such claims. All work, carefully controlled and on sufficiently large series of specimens (not on artificial mixtures of tubercle bacilli) would indicate that the actual number of positive results in guinea pigs is higher than on culture media, even though in a few instances cultures are obtained while a guinea pig inoculated with the same material fails to develop tuberculosis. For example, Stadnichenko and Sweany[49] found in a series of 200 specimens, 33 positive by animal inoculation and negative by culture, and 3 positive by culture and negative by animal inoculation.

Our own results may be mentioned in some detail, as follows: A series of 37 sputa from the Wm. H. Maybury Sanatorium in Northville was examined. All these sputa came from patients who had pulmonary tuberculosis and who had at some time had positive sputum. At the time of examination these sputa were negative on direct smear and had been so for at least one preceding examination. These 37 specimens were cultured and inoculated into guinea pigs.

In a second series of 55 specimens, including sputum, feces, exudates, tissues, urine, all negative on direct smear, the same type of study was performed. Table I summarizes the results.

TABLE I

A COMPARISON OF CULTURE AND GUINEA PIG INOCULATION IN 92 SPECIMENS FROM TUBERCULOUS PATIENTS*

NUMBER OF SPECIMENS	POSITIVE ON CULTURE (PER CENT)	POSITIVE ON ANIMAL INOCULATION (PER CENT)	CULTURE POSITIVE; GUINEA PIG NEGATIVE	GUINEA PIG POSITIVE; CULTURE NEGATIVE	POSITIVE BY ANY ONE METHOD (PER CENT)
37	78.3	89.1	4	8	100.0
55	65.4	96.3	2	19	100.0
Total 92	70.6	93.5	6	27	100.0

*With the technical assistance of A. B. Mills and R. G. Kelly.

On this series of 92 specimens it was determined how many culture tubes were positive, sterile, and contaminated. The results follow:

Total number of tubes: 710
Number of positive tubes: 338, or 47.6 per cent
Number of sterile tubes: 354, or 49.9 per cent
Number of contaminated tubes: 18, or 2.5 per cent

In the Desert Sanatorium in Tucson, a total of 238 specimens (sputa, urine, exudates, tissues) was examined, which were negative on direct smears. All of these were studied by concentration methods, cultures, and guinea pig inoculation. Table II shows the results.

TABLE II

A COMPARISON OF CULTURE AND GUINEA PIG INOCULATION IN 238 MISCELLANEOUS SPECIMENS*

NUMBER OF SPECIMENS (NEGATIVE ON DIRECT SMEAR)	POSITIVE AFTER CONCENTRATION	POSITIVE ON CULTURE	POSITIVE ON ANIMAL INOCULATION	POSITIVE BY ANY ONE METHOD	CULTURE POSITIVE; GUINEA PIG NEGATIVE	GUINEA PIG POSITIVE; CULTURE NEGATIVE
238	9	39	52	56	4	17

*With the technical assistance of J. R. Mote.

In the 56 positive specimens in Table II, it was ascertained how soon a positive diagnosis could be made by cultural methods and by animal inoculation. Table III shows the percentage of positive diagnosis made at ten days' interval.

TABLE III

A COMPARISON OF CULTURE AND GUINEA PIG INOCULATION IN REGARD TO THE SPEED OF DIAGNOSIS. ACCUMULATIVE PERCENTAGES OF POSITIVE DIAGNOSIS OBTAINED IN 10-DAY PERIODS

DAYS	-10	11-20	21-30	31-40	41-50	51-60
CULTURE	7.7	33.3	71.7	81.9	89.6	97.3
GUINEA PIG		0	30.7	63.4	86.5	94.3

Summarizing, the advantages of cultural methods are: 1, independence of animals; 2, lower cost; 3, greater speed of diagnosis, and 4, freedom of interference by premature death of animals.

On the other hand, the percentage of positive diagnoses is smaller than by animal inoculation. A complete sputum examination should include both cultural methods and animal inoculation.

5. ANIMAL INOCULATION

For animal inoculation the sputum is prepared in exactly the same way as for culture. Any of the methods, antiformin, sodium hydroxide, or acids, may be used. The sediment must be neutralized in order to avoid necroses at the site of injection. The neutralized sediment is suspended in 1 to 2 c.c. of sterile saline solution. If possible, each specimen should be injected into two animals. The best way of injection is the subcutaneous route. Other types of injection, intraperitoneal, intrahepatic, intracerebral, which have been advised in order to hasten the development of tuberculosis, have definite disadvantages; namely, a greater early mortality from trauma and secondary infection, and the impossibility of watching the local lesion develop. The injection should be made in the region of the groin, because of the proximity of lymph nodes and because the infection should be started sufficiently distant from the middle line so that the first lymph node involvement is strictly unilateral.

A number of methods designed to hasten the development of tuberculosis have been given fair trials by a number of workers, including myself. All these methods seem not to attain their stated purpose to any appreciable extent.

It is frequently discussed whether guinea pigs used for inoculation should be tested with tuberculin before infection, to ascertain that they are not spontaneously infected with tubercle bacilli. We believe this to be quite unnecessary for two good reasons: (1) notwithstanding a number of reports to the contrary, we believe that spontaneous tuberculosis in guinea pigs, kept under reasonably sanitary conditions of food and shelter, is extremely rare; (2) the autopic findings in a tuberculous guinea pig will establish beyond the possibilities of a doubt whether a spontaneous or an inoculation tuberculosis is present. It is probably one of the best established facts in experimental tuberculosis that at the site of first infection a local lesion develops and that the infection always spreads hence to the next draining lymph node. A guinea pig which shows tuberculous foci in the internal organs, but no primary lesion at the site of injection and in the regional lymph node, has acquired its tuberculosis not by injection, but by some other channel. It appears quite impossible that a spontaneous infection should enter by way of the subcutaneous tissue in the groin. Hence, a positive report of an animal inoculation should always specify that an *inoculation*-tuberculosis was found.

When should the autopsy be performed on an inoculated guinea pig? Starting about two weeks after inoculation, the animal should be examined at intervals for the appearance of the local lesion and the swelling of the deep inguinal lymph node. As soon as a local lesion or enlarged nodes are demonstrable, necrotic tissue from the inoculation ulcer, or an excised node may be examined in

smears stained for acid-fast bacilli. If they are found, the animal may be autopsied to confirm the preliminary diagnosis. If no acid-fast rods are seen, the animal should be kept alive for further observation.

Unless definite signs of tuberculosis (the local lesion!) develop, no animal should be killed sooner than eight weeks after inoculation. No inoculated animal should be reported "negative" without complete autoptic examination. If one chooses one may perform tuberculin tests (intracutaneous) on inoculated animals, starting about fifteen days after infection; this may help to arrive at an earlier diagnosis; but a positive tuberculin test without autopsy should never be accepted as final proof; nor should repeatedly negative tuberculin tests be accepted as full evidence for the absence of tuberculosis. Animals dying within the first three weeks after inoculation and showing no tuberculous lesions, should not be reported as negative. In such animals, tubercle bacilli or specific tissue changes may be found on microscopic examination.

6. THE CLINICAL SIGNIFICANCE OF POSITIVE AND NEGATIVE SPUTUM

The following remarks apply only to the results of thorough and complete examinations. By this is meant: (1) a careful search for acid-fast rods in technically flawless smears, each search to last about ten minutes, if necessary; (2) frequent repetitions of such examinations, using, if necessary, sputum collected for several days; (3) the use of concentration methods, of cultural methods, and of animal inoculation.

Provided that these requirements be fulfilled both the finding of tubercle bacilli and the failure of finding them, constitute laboratory data of such high reliability as to be rivalled by few laboratory procedures. In a study from the Maybury Sanatorium, Pinner and Werner[45] reported on sputum examinations on a consecutive series of 585 patients; of these 14 had nontuberculous pulmonary lesions, 33 had no pulmonary lesions, leaving a total of 538 patients with the clinical diagnosis of pulmonary tuberculosis. Of this group of patients, 36 had negative sputum in spite of exhaustive studies on their sputa. Clinically, these 36 negative sputum patients were classified as follows:

Apparently cured,	3
Apparently arrested,	20
Quiescent,	8
Active,	5

In a group of 507 patients with clinically active pulmonary tuberculosis, tubercle bacilli were demonstrated in 502, or a fraction more than 99 per cent. On the basis of such evidence, is it not a judicious claim that the failure to demonstrate tubercle bacilli in a patient's sputum should not be disregarded lightly, as much of the routine teaching advises? Is it not reasonable to ascribe to such negative findings a considerable diagnostic or prognostic value? Should negative findings not be considered as indicating the likelihood of either a healed or healing tuberculous focus, or of a nontuberculous pulmonary disease? Without trying to establish a hard and fast rule, we have found that the following is a practical working principle. If a patient has a demonstrable pulmonary lesion and

such symptoms as are consistent with that lesion; if, in addition, he has a sizeable amount of sputum (say 10 c.c. per day or more), then, the failure to find tubercle bacilli in his sputum should put the burden of proof on the shoulders of him who claims tuberculosis to be the etiologic factor. Such considerations become of special weight if the laboratory data are properly correlated with clinical findings. Although roentgenologic observations can, strictly speaking, never decide the etiology of a pulmonary lesion, the localization, upper or lower portions of the lung fields, gives indications which in conjunction with laboratory data enable the clinician at times to arrive at a definite diagnosis without strictly positive evidence.

What of a positive sputum? If we can be sure of the uncontaminated source of sputum, excluding malingering with its not infrequent attempts at falsification in the matter of sputum, positive findings constitute a prima facie evidence of pulmonary tuberculosis. Possible errors are extremely rare; but when positive laboratory findings clash with negative clinical findings, two possible sources of error must be considered: 1. Acid-fast bacilli demonstrated in the sputum may be apathogenic organisms and not tubercle bacilli. A few such findings are reported in the literature. Pappenheim found large numbers of acid-fast bacilli, which he diagnosed as smegma bacilli, in a case of multiple pulmonary abscesses and bronchiectases. Similar findings are reported by Rabinowitsch,[46] who was able to cultivate these acid-fast apathogens. Since they grew in twenty-four hours to visible colonies, the differential diagnosis was easily established. Nonpathogenic acid-fast bacilli were demonstrated in the sputum in gangrene by Fränkel,[16] in bronchitis fibrinosa by Lichtenstein,[32] in bronchiectasis by Milchner.[37] Alexander[1] isolated acid-fast rods from the nasal secretion in ozena, Karlinsky[25] and Laabs[31] from nasal secretions in normal persons, Möller[38] from saliva in normal persons. It must, of course, be remembered that the leprosy bacillus and certain elements, particularly spores of higher bacilli, are acid-fast.

From my own experience, I can quote but one instance in which nonpathogenic acid-fast rods were found in sputum. A young girl with a valvular heart lesion expectorated sputum twelve hours before death in which typical acid-fast rods were demonstrated on several smears. The autopsy, next day, did not reveal any sign of tuberculosis. A guinea pig injected with that sputum was killed more than ten weeks after the inoculation and was found to be grossly normal. But smears from the lymph node at the site of injection revealed numerous acid-fast rods.

The exact source of nonpathogenic acid-fast rods in human secretions is probably impossible to determine on account of the widespread occurrence of such organisms in dairy products, grass, manure, and water. The latter source must be kept in mind as a possible contamination during laboratory procedures. The cultural characteristic of some of the apathogens may be diagnostic; but a distinction by mere tinctorial and morphologic characteristics in smear preparations is unreliable.

To differentiate true tubercle bacilli from other members of the acid-fast group, it is best to use animal inoculation. If a specimen in which acid-fast organisms are demonstrated fails to infect a guinea pig, pure cultures should be

obtained of such doubtful acid-fast organisms, and animal inoculations should be repeated with pure cultures.

2. The possibility must be kept in mind that tubercle bacilli in sputum may be derived not only from a pulmonary focus, but also from a tuberculous lesion in the upper respiratory tract. Such lesions in the absence of pulmonary tuberculosis are exceedingly rare. I saw a young women from whose sputum a pure culture of tubercle bacilli was isolated. Clinically and roentgenologically, there was not the slightest sign of a pulmonary lesion. Her tonsils were removed and showed, on histologic examination, multiple caseated tubercles. In the absence of all evidence of a pulmonary lesion, with positive sputum findings, and with or without symptoms suggestive of tuberculosis, the possibility of a lesion in a hilar lymph node, ruptured into a bronchus or the trachea, must be considered. That such ruptured lesions may exist without producing parenchymal involvement, was shown by a case in which such a rupture, scarred at the time of autopsy, existed and in which no trace of pulmonary tuberculosis, barring an encapsulated primary focus, was found.

These two sources of error are mentioned here not in order to point out their importance, but to emphasize their extreme rarity. On the other hand, it should not be forgotten that Löwenstein[33] has mentioned repeatedly that he has seen a few cases in which tubercle bacilli from proved tuberculous lesions in man, were apathogenic for guinea pigs.

The avian tubercle bacillus is known to infect man on rare occasions. In a recent review, Branch[4] collected 15 cases from the literature; in only one of them were avian tubercle bacilli found in the sputum; the other 14 patients had exclusively or mainly extrapulmonary lesions. Avian tubercle bacilli are pathogenic for rabbits, producing on intravenous inoculation, usually a septicemia; they are pathogenic for fowl, but in the usual dosage apathogenic for guinea pigs. The differentiation must be made on this basis of species pathogenicity.

The literature is replete with attempts to find prognostic indications in the number of bacilli and in their morphologic characteristics. First as to the number: the bacilli, as mentioned, are quite irregularly distributed throughout the sputum; a minute random sample can, therefore, not be considered as representative of the entire specimen. Any attempt at a quantitative evaluation seems totally unjustified. The well-known Gaffky scale, with six or ten classes in regard to the number of bacilli, claims a much finer distinction than is actually possible on the basis of the technic employed. A very rough classification, such as: "very few," "moderate number," "very many," or simpler +, ++, +++, ++++, may have a limited justification. A continuous decline in the number of bacilli may be regarded as a possible favorable sign, but occasional expectorations containing innumerable bacilli may be without prognostic significance; they are not rare occurrences in quiescent lesions.

If, for some reason, one wishes to obtain an accurate idea of the number of bacilli present, actual counting must be done. Butschowitz[5] published a procedure for this purpose.

As to the shape of bacilli: long or short, thick or thin, solid or beaded, I know of no convincing evidence that—as has been claimed—such variations in shape have any bearing on prognosis.

The number of diagnostic and prognostic laboratory tests for tuberculosis is appalling: specific and nonspecific serologic tests, blood chemical data, differential blood counts, ferment reactions of the serum, etc.; they all have had their supporters. But, may it be stated emphatically in conclusion, that the clinical laboratory has no more important aid to offer to the clinician in practical work in pulmonary tuberculosis than thorough and complete bacteriologic studies of the sputum.

REFERENCES

1. Alexander: Über säurefeste Bazillen im Ozänasekret, Berl. klin. Wchnschr. **40**: 508, 1903.
2. Armand-Delille, P. F., and Vibert, J.: Le diagnostic bactériologigue de la tuberculose pulmonaire des jeunes enfants par l'examen du contenu gastrique, Bull. Acad. de méd., Paris **97**: 373, 1927.
3. Biedert: Ein Verfahren, den Nachweis vereinzelter Tuberkelbazillen zu sichern, nebst Bemerkungen über die Färbbarkeit der Bazillen und Aetiologie der Tuberkulose, Berl. klin. Wchnschr. **23**: 713, 1886.
4. Branch, A.: Avian Tubercle Bacillus Infection With Special Reference to Mammals and to Man, Arch. Path. **12**: 253, 1931.
5. Butschowitz, E.: Methode zur quantitativen Bestimmung der toten und lebenden Tuberkelbazillen im Phthisikersputum, Ztschr. f. Tuberk. **60**: 149, 1931.
6. Capaldi, A.: Zur Verwendung des Eidotters als Nährbodenzusatz, Centralbl. f. Bakteriol. **20**: 800, 1896.
7. Cooper, F. B.: A Modification of the Ziehl-Neelsen Staining Method for Tubercle Bacilli, Arch. Path. **2**: 382, 1926.
8. Corper, H. J.: Methods of Staining Tubercle Bacilli, J. Lab. & Clin. Med. **11**: 3, 1926.
9. Corper, H. J., and Uyei, N.: The Cultivation of Tubercle Bacilli. An Improved Method for Isolation From Tuberculous Materials, J. Lab. & Clin. Med. **13**: 469, 1928.
10. Corper, H. J.: The Certified Diagnosis of Tuberculosis, J. A. M. A. **91**: 371, 1928.
11. Corper, H. J., and Uyei, N.: A Simple Glycerol Water Crystal Violet Potato Cylinder Medium for Diagnostic Cultures of Tubercle Bacilli, Arch. Path. **7**: 835, 1929.
12. Corper, H. J., and Uyei, N.: Further Observations With a New Method for Cultivating Tubercle Bacilli: A Comparison With Guinea Pig Inoculation and Petroff's Method, J. Lab. & Clin. Med. **14**: 393, 1929.
13. Corper, H. J., and Uyei, N.: Oxalic Acid as a Reagent for Isolating Tubercle Bacilli and a Study of the Growth of Acid-fast Nonpathogens on Different Media With Their Reaction to Chemical Reagents, J. Lab. & Clin. Med. **15**: 348, 1930.
14. Czaplewsky and Hensel: Zum Nachweis der Tuberkelbazillen im Sputum, Ztschr. f. Tuberk. **1**: 387, 1900.
15. Dorset, M.: The Use of Eggs as a Medium for the Cultivation of Bacillus Tuberculosis, Am. Med. **3**: 55, 1902.
16. Fränkel, A.: Sekundäre Infection mit Tuberkelbazillen, Berl. klin. Wchnschr. **35**: 246, 1898.
17. Gabbet, H. S.: Rapid Staining of the Tubercle Bacillus, Lancet **1**: 757, 1887.
18. Herman, M.: Procédé rapide de coloration du bacille tuberculeux, Ann. de l'Inst. Pasteur **3**: 100, 1889.
19. Herman, M.: Sur la coloration du bacille tuberculeux, Ann. de l'Inst. Pasteur **12**: 92, 1908.
20. Herrold, R. D.: Egg Yolk Agar Medium for the Growth of Tubercle Bacilli, J. Infect. Dis. **48**: 236, 1931.
21. Hohn, J.: Kultur der Tuberkelbazillen, Centralbl. f. Bakteriol. **98**: 460, 1926.
22. Hohn, J.: Zur Frage der Kultur der Tuberkelbazillen und ihrer Verwendung zur Diagnose der Tuberkulose, Centralbl. f. Bakteriol. **103**: 342, 1927.
23. Hohn, J.: Vierjährige Erfahrung mit der Kultur des Tuberkelbazillus zur Diagnose der Tuberkulose, München. med. Wchnschr. **76**: 1120, 1508, 1929.

23a. Hohn, J.: Der Z- Einährboden zur Kultur des Tuberkelbazillus, Centralbl. f. Bakteriol. **121**: 488, 1931.

24. Jessen, F.: Gleichzeitiger Nachweis von Tuberkelbazillen und elastischen Fasern in demselben Praeparat, Beitr. z. klin. d. Tuberk. **65**: 4, 1926.
25. Karlinsky, Y.: Zur Kenntnis der säurefesten Bakterien, Centralbl. f. Bakteriol. **29**: 521, 1901.
26. Kiefer, J.: A New and Easy Method for the Demonstration of Granules in Tubercle Bacilli, Am. Rev. Tuberc. **5**: 662, 1921.
27. Koch, R.: Die Aetiologie der Tuberkulose, Berl. klin. Wchnschr. **29**: 221, 1882.
28. Konrich: Eine neue Färbung für Tuberkelbazillen, Deutsche med. Wchnschr. **46**: 741, 1920.

29. Kitasato, S.: Gewinnung von Reinculturen der Tuberkelbacillen und andrer pathogener Bacterien aus Sputum, Ztschr. f. Hyg. **11**: 441, 1892.
30. Krause, A. K.: Remarks on the Laboratory Diagnosis of Pulmonary Tuberculosis, Am. Rev. Tubere. **18**: 51, 1928.
31. Laabs: Über tuberkelbazillenähnliche Stäbchen in verschiedenen Körpersekreten. Inaug.-Dissert., Freiburg, 1894.
32. Lichtenstein, E.: Über das Vorkommen von Pseudotuberkelbazillen im menschlichen Sputum, Ztschr. f. Tuberk. **3**: 193, 1902.
33. Löwenstein, E.: Vorlesungen über Tuberkulose, Jena, 1920.
34. Löwenstein, E.: Beitrag zur Leistungsfähigkeit der direkten Züchtung der Tuberkelbazillen aus infectiösem Material, Wien. klin. Wchnschr. **37**: 231, 1924.
34a. Löwenstein, E.: Die Züchtung der Tuberkelbazillen aus dem strömenden Blute, Centralbl. f. Bakteriol. **120**: 127, 1931.
35. Lubenau, C.: Der Eigelbnährboden als Ersatz des Serums zur Kultur von Diphtherie und Tuberkelbacillen, Hyg. Rundschau **17**: 1455, 1907.
36. Matzuschita, T.: Über die Wachstumsunterschiede der Bacillen der Hühnertuberkulose und der menschlichen Tuberkulose auf pflanzlichen, Gelatine-und Agarnährböden, Centralbl. f. Bakteriol. **26**: 125, 1899.
37. Milchner, R.: Über Pseudotuberkelbacillen in einem Falle hochgradiger Bronchiektasie und ihr Verhalten gegenüber Farbstoffen, Deutsche med. Wchnschr. **29**: 130, 1903.
38. Möller: Zur Verbreitungsweise der Tuberkelpilze, Ztschr. f. Hyg. **32**: 205, 1889.
39. Mühlhäuser: Über das Biedertsche Verfahren zum Nachweis von Tuberkelbazillen, Deutsche med. Wchnschr. **17**: 282, 1891.
40. Nocard and Roux: Sur la culture du bacille de la tuberculose, Ann. de l'Inst. Pasteur **1**: 19, 1888.
41. Pappenheim, A.: Befund von Smegmabazillen im menschlichen Auswurf, Berl. klin. Wchnschr. **35**: 809, 1898.
42. Pawlowsky, A. D.: Culture des bacilles de la tuberculose sur la pomme de terre, Ann. de l'Inst. Pasteur **2**: 303, 1888.
43. Petragnani, G.: Terreno e tecnica per l'isolamento in cultura pura dei B. di Koch dagli escreati e da altri materiali tubercolari, Atti d. r. Accad. d. fisiocrit. in Siena **1**: 177, 1926.
44. Petroff, S. A.: A New and Rapid Method for the Isolation and Cultivation of Tubercle Bacilli Directly From the Sputum and Feces, J. Exper. Med. **21**: 38, 1915.
45. Pinner, M., and Werner, W. I.: The Significance of Positive and Negative Sputum Findings in Pulmonary Tuberculosis, Am. Rev. Tubere. **18**: 490, 1928.
46. Rabinowitsch, L.: Befund von säurefesten tuberkelbazillenähnlichen Bakterien bei Lungengangrän, Deutsche med. Wchnschr. **26**: 257, 1900.
47. Rappaport, I., and Ellison, R. T.: Tubercle Bacilli and Elastic Tissue, J. Lab. & Clin. Med. **14**: 261, 1929.
48. Spengler, C.: Neue Färbemethoden für Perlsucht-und Tuberkelbazillen und deren Differentialdiagnose, Deutsche med. Wchnschr. **33**: 337, 1907.
49. Stadnichenko, A., and Sweany, H. C.: A Comparison of Culture and Animal Inoculation of Sputum in the Diagnosis of Tuberculosis, Am. J. Clin. Path. **1**: 303, 1931.
50. Sumoyoshi, Y.: Beitrag zur Reinzüchtung von Tuberkelbazillen aus dem Sputum, Ztschr. f. Tuberk. **39**: 5, 1924.
51. Sweany, H. C., and Evanoff, M.: The Isolation of Tubercle Bacilli From Septic Material, Am. Rev. Tubere. **17**: 47, 1928.
52. Sweany, H. C.: Examination of Sputum for Acid-fast Bacilli, Arch. Path. **6**: 263, 1928.
53. Sweany, H. C., and Evanoff, M.: Further Studies on the Cultivation of the Tubercle Bacillus, Am Rev. Tubere. **18**: 661, 1928.
54. Uhlenhuth and Xylander: Antiformin, ein bakterienauflösendes Desinfektionsmittel, Berl. klin. Wchnschr. **45**: 134, 1908.
55. Weiss, L.: Zur Morphologie des Tuberkulosevirus unter besonderer Berücksichtigung der Much, schen granulären Form und einer neuen Doppelfärbung. Mitt. a. d. Hamb. Staatskrankenanst. **11**: 235, 1910.
56. Willis, H. S.: Laboratory Diagnosis and Experimental Methods in Tuberculosis, Springfield, Ill., 1928.
57. Ziehl, F.: Zur Färbung des Tuberkelbazillus, Deutsche med. Wchnschr. **8**: 451, 1882.

THE ISOLATION AND IDENTIFICATION OF PATHOGENIC BACTERIA IN FECES*

By Leon C. Havens, M.D., Montgomery, Ala.

THE detection of pathogenic bacteria in feces is one of the most complicated bacteriologic problems which a diagnostic laboratory encounters. Their isolation, in the first place, is attended by many technical pitfalls, and any significant organisms which may be found require intelligent and careful work for their identification. There are many intestinal bacteria which closely resemble the known pathogens but which so far have no definitely ascribed rôle. Their significance is uncertain and their presence is often confusing. Hence, one can hardly be too careful in the procedures used for purposes of identification. In the majority of instances, of course, such as typhoid fever, the offending organism is readily identified by comparatively simple cultural and agglutinative reactions. But when one approaches the paratyphoid group and, particularly the dysenteries, skill and experience are needed. It is here, in this group of diseases, due to their comparatively infrequent occurrence and their vague clinical symptoms, that the laboratory is called on to actually establish the diagnosis. Thus, even though such examples appear comparatively infrequently, it is, nevertheless, essential that the laboratory be prepared to make an intelligent examination. A successful attack on one obscure case of this nature may enhance the prestige and increase the reputation of a laboratory more than any number of ordinary, routine examinations.

The subject matter of this paper makes little pretense of presenting new material. Its chief purposes are (1) to bring together in convenient form methods which have been found useful and practicable for the bacteriologic examination of fecal specimens, and (2) to discuss the significance of some of the pathogens which leave the body through the intestinal tract. We make, in our Central Laboratory, of necessity, large numbers of such examinations and have had thrust upon us the evolving of methods which can readily be utilized in our branch laboratories. The practices adopted must be neither too expensive nor too time-consuming for a laboratory with limited personnel and funds and, at the same time, must give assurance of thoroughness and accuracy. This has necessitated the sifting of known methods—which are legion—and, in some instances, the development of new procedures.

One is impressed, in studying the avilable treatises of clinical diagnostic methods, by the casual manner in which the bacteriology of the feces has been handled. In fact, the following surprising statement may be found,[1] "For laboratory diagnosis (of typhoid fever) blood cultures during the first week and agglutination tests during the second week and onward are the practical methods." Probably such hasty dismissal of bacteriologic methods is due in large part to their very multiplicity. There is, perhaps, no other diagnostic procedure involving bacteriologic methods which has received less standardization

*From the Laboratories of Alabama State Board of Health, Montgomery.

than that of the intestinal tract. Almost every bacteriologist has his favorite procedures which seem to be successful in his own hands; consequently, he hesitates to accept new methods or modifications of old ones. The situation is comparable to that formerly existing in the serologic diagnosis of syphilis. One may hope, therefore, that, eventually, there may be established, for the diagnosis of intestinal pathogens, criteria which will serve a similar useful purpose in clarifying known facts and crystallizing the technical chaos.

In view of the accumulating evidence of the lack of diagnostic value of agglutination tests with the patient's serum, the extent to which the clinician still relies upon these simple procedures is regrettable. The only certain means of establishing the nature of an infection is the isolation of the causative organism. This is particularly true of the typhoid-paratyphoid-dysentery groups, where the phenomenon of cross-agglutination occurs so frequently, to say nothing of the development of agglutinins as a result of vaccination and missed or subclinical infections. It is the duty of the diagnostic laboratory to educate the clinician in the proper use of the laboratory; encouragement in the routine use of cultural methods must presuppose adequate equipment, mental as well as physical, on the part of the laboratory itself!

From our standpoint, the important bacteria of the intestinal tract are B. typhosus, the Salmonella group, and the dysenteries. The first problem in the bacteriologic examination of any intestinal infection is assurance of a dependable specimen with which to work. Even more than with other laboratory examinations a successful result is governed largely by the intelligence and care used in the collection of the specimen, together with the promptness and skill attending the initial steps in the examination. Unless the feces can be immediately taken to the laboratory and cultures made without delay, as in a hospital, special methods for preservation are necessary. Even under ideal conditions, some means of preserving the specimen for further study are desirable, since, as will appear later, repeated platings materially increase the percentage of positive results.

For these reasons, a large amount of study has been focused on the treatment of the specimen prior to the bacteriologic examination. The chief effort, of course, is to prevent or retard the enormous overgrowth of B. coli, which occurs so rapidly in untreated feces.[2] Most of the methods depend upon the use of brilliant green. In the description of the technic which follows, we have utilized the sharp selective action of this dye for the preservation of the specimen itself, rather than the customary usage in preliminary enrichment cultures. Since the dye has no selective action on the dysentery bacilli, other methods must be used for their isolation. This is one of the chief reasons for the difficulty attending the isolation of members of this group.

PREPARATION OF MEDIA USED IN ISOLATION AND IDENTIFICATION

In this description of methods of isolation and identification we have included details only of those procedures not readily found in most textbooks of bacteriology. We have not given the formulas, for example, for plain broth and Russell's double sugar agar, because they are readily available elsewhere, but, on the other hand, we have included the details of such media as Simmons'

citrate agar and Jordan's tartrate medium, not only because they are comparatively new, but also because their usefulness in the differentiation of intestinal bacteria warrants wider application in routine diagnostic practice.

Repeated plating of the specimen is another procedure, the importance of which should be stressed. It not infrequently happens that a positive result is obtained only on the second or even the third day plates, after further diminution in the total bacterial flora has occurred, and, as in the case of brilliant green bile cultures, an actual increase in the numbers of typhoid or paratyphoid colonies has taken place.

Brilliant Green Solution.—The brilliant green at present obtainable is much less satisfactory for the isolation of typhoid bacilli from feces than that formerly obtainable, owing to a decrease in its selective action. While batches of the dye can be found that are fairly suitable for brilliant green agar, it has been impossible to obtain any recent dye that will serve the purpose in bile. For this reason attempts were made to alter or modify the dye in the hope that its selective action could thereby be increased.

The following treatment has been found, in most instances, to increase greatly the selective action of brilliant green: A 5 per cent solution of the dye in N/3 hydrochloric acid is evaporated to a syrupy consistency* over a boiling water-bath. The evaporated dye is redissolved in distilled water to make a 5 per cent solution and kept for use. When the dye is dissolved in the acid, a color change takes place—a yellow or burnt orange—but the final color is again green.

The proper concentration of the treated dye is determined by titration. Fifteen c.c. of bile (Digestive Ferments Co., dehydrated bile) is placed in each of a series of 1 oz. bottles. To the first bottle is added 2.5 c.c. of the 5 per cent stock solution (concentration 1: 120); to the second, 2.0 c.c. (1: 150), and decreasing amounts to the succeeding bottles to a concentration of 1: 300 or 1: 400, with intervals between the concentrations of not more than 20 to 25 per cent. In our experience the greatest selective action falls within this range, usually around 1: 200. Several (4-6) sets covering this range should be prepared to permit testing with several specimens of feces. The bottles are labelled and sterilized in the autoclave.

To each bottle is then added 1 c.c. of a 1: 10 suspension of feces in salt solution and 0.1 c.c. of a twenty-four-hour broth culture of B. typhosus. The bottles are kept at room temperature† and a loopful from each streaked on brilliant green agar plates at the end of each twenty-four-hour interval for three days. That concentration of brilliant green is used, for the particular batch of bile in question, which gives the greatest yield of typhoid colonies, together with the most pronounced inhibition of fecal bacteria.

This titration is absolutely essential to the success of the method. It is advisable, also, as a further check, to test the finished batch of brilliant green bile with several different specimens of feces seeded with typhoid. This medium is satisfactory for blood and urine cultures, as well as for feces.

The brilliant green agar used for plating must also be carefully titrated. We prefer brilliant green Endo agar, as it gives, in our hands, better differentiation between lactose- and nonlactose-fermenting colonies. Whether Krumwiede's agar is used or brilliant green Endo agar, the proper concentration of dye is determined for each lot of agar.[3]

It cannot be emphasized too strongly that the success of the method lies in carefully determining the proper concentration of the dye for each new batch of brilliant green, each new batch of bile, and each new batch of agar. It is possible with this method to obtain, with many specimens of feces, almost pure cultures of typhoid for several days after the preparation of the specimen.

---

*The end-point described for the evaporation process seems somewhat indefinite but as a matter of fact, there is a fairly wide range within which the action of the dye is satisfactory. If evaporation is stopped before the syrupy consistency is reached, the resulting solution is inhibitory to B. typhosus, while if carried to a gummy consistency, it is no longer inhibitory to B. coli. Between these points, batches of dye with reasonably good selective action can almost always be obtained.

†Comparative studies at 20° C. and 37° C. indicate that the selective action of the dye is much less pronounced at the higher temperature, with consequent increase in numbers of B. coli.

Preparation of Bile Medium.—Dissolve 100 grams Bacto-oxgall (Digestive Ferments Co.) in 1000 c.c. distilled water, and sterilize for thirty minutes at 15 pounds pressure. The Digestive Ferments Co. labels bear a number. All bile of the same number may be used without additional titrations for the proper brilliant green content.

Preparation of Brilliant Green Endo Agar.—Forty-one and five-tenths grams of dehydrated Endo agar (Digestive Ferments Co.) are dissolved in 1000 c.c. of water, and sterilized for fifteen minutes at 15 pounds pressure. The medium is then given a lot number, titrated, and placed in the cold room until ready for use. Each reference number on the label of the bottle of dehydrated agar is noted and each new lot is numbered.

Titration.—Five flasks, each containing 200 c.c. of Endo medium, are melted, and to these are added increasing concentrations of the 5 per cent brilliant green solution covering the range within which the proper dilution lies (this is usually between 1:100,000 and 1:250,000).

A plate of each dilution is then streaked for two successive days with a loopful from a brilliant green bile culture prepared twenty-four hours before by adding 0.1 c.c. of a twenty-four-hour broth culture of B. typhosus and 1.0 c.c. of 1:10 dilution of feces in salt solution to 15 c.c. of brilliant green bile. That concentration is chosen which gives the most complete inhibition of fecal bacteria, and at the same time no marked inhibition of B. typhosus, as evidenced by the number and size of the colonies.

It is advisable to set up three or more such bottles using a different specimen of feces in each, since some feces contain organisms not readily inhibited by the dye and others may yield almost pure cultures of B. typhosus; in either case, consequently, a clear-cut titration is not obtained.

Preparation of Sugar Broth.—Prepare plain beef extract broth, to which is added 1 per cent Andrade indicator (reaction adjusted to indicator—P_H 7.2-7.4). Place in small tubes (½″ by 4″) in 2 c.c. amounts with fermentation tubes* and sterilize for thirty minutes at 15 pounds pressure. Then add aseptically the sugar solutions (0.05 c.c. of 20 per cent solution, to make 0.5 per cent). Mark sugars by coloring cotton plugs with different dyes as a means of identification and sterilize in Arnold sterilizer for sixty minutes. Incubate for sterility and test with known stock cultures.

Stock Sugar Solutions.—Weigh accurately 1 gram of the sugar and dissolve in 5 c.c. of distilled water (making a 20 per cent solution). Sterilize for twenty minutes at 15 pounds pressure.

SODIUM POTASSIUM TARTRATE MEDIUM[4]

Agar	20 grams
Distilled water	1000 c.c.
Alcohol sol. phenol red (2 per cent)	12 c.c.
Difco peptone	10 grams
Sodium potassium tartrate	10 grams
Sodium chloride	5 grams

*These are conveniently made by sealing 6 to 8 mm. tubing at the end and cutting off in 10 to 12 mm. lengths.

Adjust medium to P_H 7.6-7.8, tube, sterilize for twenty minutes at 15 pounds pressure and slant. Incubate for sterility and test with known cultures. Store in the cold room. It has been our observation that the medium should be fresh. Tubes which have been made for some time (two weeks or longer), even though stored in the ice box, fail to give sharp reactions.

SIMMONS' CITRATE AGAR[5]

Agar	20.0	grams
NaCl	5.0	grams
$MgSO_4$	0.2	grams
(NH_4) H_2PO_4	1.0	grams
K_2H PO_4	1.0	grams
Sodium citrate (anhydrous)	2.0	grams
or ($5\frac{1}{2}$ H_2O)	2.77	grams
Distilled water	1000	c.c.
Bromthymol blue (1.5 per cent in 95 per cent alcohol)	10	c.c.

Adjust to P_H 6.8, add indicator, tube, sterilize for twenty minutes at 15 pounds pressure, and slant. Incubate over night for sterility, test with known cultures, and place in cold room.

Collection of the Specimen.—The brilliant green bile is used for blood (5 to 10 c.c.), urine (10 to 15 c.c.), and feces (about 0.1 gram or about the size of a small bean). It is very important to give instructions that only a small amount of feces be added, as larger amounts will absorb so much of the dye that overgrowth by fecal bacteria results.

Isolation and Identification.—For the typhoid-paratyphoid group, specimens are received into the laboratory in brilliant green bile. For the dysenteries the usual 30 per cent glycerin in 0.85 per cent NaCl is employed. Usually, upwards of twenty-four hours elapse after collection before the specimen is examined. The specimens, thus preserved, are streaked on three succeeding days on brilliant green Endo agar and on plain Endo plates, one loopful from the surface of each bottle being used, without agitation of the contents, the bottles being left at room temperature in the meantime. The purpose of the three successive platings is to permit multiplication, in the bile, of original small numbers of organisms to a point where they may be detected on a plate streaked with one loopful of the culture, and to permit further progressive decrease in the numbers of B. coli and other fecal bacteria which interfere.

A macroscopic slide agglutination test is made from characteristic colonies on the plates. This gives preliminary evidence of the nature of the organism. If the colony is typical and if the performance of the particular serum and the dilution in which it is used are known by constant daily experience, a preliminary report of specimens from clinical cases may be immediately made on the basis of this test alone.* If conservatively used, it is invariably confirmed by the subsequent fermentation and agglutination tests.

*The test is performed by placing a loopful of the appropriate serum dilution on the slide. Touch the point of the needle into the colony to be tested and emulsify in the loopful of serum. A positive test is indicated by the formation of large clumps, plainly visible to the naked eye. Each serum used for the macroscopic slide agglutination test must be titrated against a number of closely related organisms (e. g., B. typhosus, paratyphosus A and B, B. morgani), and the lowest dilution used which gives definite, rapid agglutination (in from ten to fifteen seconds) with its homologous strain and no cross-agglutination with the closely related strains. (See Park and Williams: Pathogenic Microorganisms, Philadelphia, ed. 8, p. 204.) The clumps form, usually, almost at once. If agglutination is delayed or is incomplete, it should be interpreted with caution.

Regardless of the results of the slide agglutination, suspicious colonies are transferred to Russell double sugar tubes. From the Russell tubes fermentation tests are made on the following carbohydrates: adonite, mannite, rhamnose, saccharose, and sorbite. These five fermentable substances have been found particularly useful for purposes of identification. Adonite is, perhaps, the least essential, but it often serves to detect certain members of the colon group which yield colorless colonies on Endo after twenty-four hours' incubation.

In addition to the carbohydrate media, tubes of citrate agar, tartrate agar, and peptone water (for indol test) are inoculated.

At the same time, a plain agar slant is inoculated for a macroscopic tube agglutination test, and the culture (Russell tube) from which the sugar broths are inoculated is emulsified in peptone water and a loopful from this streaked on Endo plates. This last step gives valuable information regarding the characteristics of the colonies in pure culture, and constitute a check upon the purity of the culture in the carbohydrate media.

We have found this "re-streak" plate particularly useful as an aid in classification. Besides the colony characteristics and the assurance of a pure culture

TABLE I

CULTURAL REACTIONS OF NONLACTOSE-FERMENTING INTESTINAL BACTERIA

CULTURE	RUSSELL DOUBLE SUGAR AGAR	CARBOHYDRATE REACTIONS					INDOL	CITRATE	TARTRATE
		ADONITE	MANNITE	RHAMNOSE	SACCHAROSE	SORBITE			
B. typhosus	+	−	+	−	−	+	−	−	A
B. paratyphosus A	⊕	−	⊕	+	−	⊕	−	−	−
B. paratyphosus B (Schottmüller)	⊕	−	⊕	⊕	−	⊕	−	+	−
B. paratyphosus B (Aertrycke)	⊕	−	⊕	⊕	−	⊕	−	+	A
B. morgani	+ or ⊕	−	−	−	− or ⊕ S	−	+	−	AA
B. enteritidis	⊕	−	⊕	⊕	−	⊕	−	+	A
B. suipestifer (S. cholerae suis)	⊕	−	⊕	⊕	−	⊕	−	−	A
B. dysenteriae (Shiga)	+	−	−	−	−	−	−	−	AA
B. dysenteriae (Flexner)	+	−	+	+	−	+	+	−	A
B. dysenteriae (Strong)	+	−	+	−	+	−	+	−	A
B. dysenteriae (Y)	+	−	+	−	−	−	+	−	A
B. dysenteriae (Sonne)*	+	−	+	+	+ S	−	−	−	A
B. proteus	⊕	−	−	−	⊕	−	+	+	AA

—, No acid or gas; +, acid; ⊕, acid and gas; A, acid butt; AA, acid butt, acid slant; S, slow fermentation (7-10 days); *, ferments lactose slowly.

for further study, this second plating from the Russell tubes frequently gives clear evidence of slow lactose-fermenting strains. Although the colonies on the original plate may have been colorless and the reaction on Russell medium typical of a Salmonella organism, when plated again on Endo, such cultures often ferment lactose rapidly. There is a consequent saving of time in further identification which might have been attempted, had this single step been omitted.

If a strain is isolated which gives typical cultural reactions but which does not agglutinate with the homologous monovalent serum indicated by the carbohydrate reactions, it is subcultured daily on plain agar slants. Usually six or eight transfers are sufficient to render it agglutinable. Strains isolated from the feces are, in our experience, not often inagglutinable. Blood cultures, on the other hand, frequently show inagglutinable colonies on the plate (as evidenced by slide agglutination). The great majority of these become agglutinable as soon as they have been transferred to Russell tubes.

INTERPRETATION OF RESULTS

In the foregoing description of methods of identification too much significance should not be attached to minor variations. For example, Morgan's bacillus may ferment saccharose slowly.[6] A number of strains in our collection have been found to possess this characteristic. It is, of course, well known that freshly isolated cultures of the dysentery group may give atypical reactions. Agglutination with known monovalent serums must always be the final test, particularly after several transfers on artificial media. The serological characteristics, as well as the cultural, tend to become stable, as a rule, after a few subcultures.

A useful lead to the significance of any unusual organism isolated from the feces may be gained from an agglutination test with the patient's serum. This is particularly true of the paradysenteries and certain of the Salmonellas which are not only difficult to classify without extended study, but are known to occur sometimes in the healthy intestine. Tests with the patient's serum have been particularly useful, in our own experience, in determining the significance of Morgan's bacillus. This organism has been reported[7] as the cause of clinical paratyphoid fever, but since it is not infrequently found in normal stools, its isolation alone is not sufficient to establish it as the cause of the infection. In a series of cases which we have studied,[8] we found that agglutinins were invariably produced against the homologous culture and that the agglutinin titer rose during the course of the infection. That an organism can be shown to have caused a reaction on the part of the host, and, further, that this reaction is an increasing one, as evidenced by a subsequent stronger agglutination, constitutes strong presumptive evidence that it is the cause of the clinical manifestations.

SPECIFIC CONSIDERATIONS

Typhoid.—The isolation of B. typhosus from the feces is sufficient, coupled with typical clinical symptoms, to establish the diagnosis. With atypical clini-

cal symptoms, a positive finding in the feces must be qualified by the possibility of the carrier condition.

One of the chief objections to cultural methods, on the part of clinicians, is the delay in obtaining the result. A preliminary report, based on colony agglutination, may often be given within twenty-four hours. If the colony is typical* in appearance and specific agglutination occurs, a preliminary report is justified, with no more delay than with a simple Widal test. It cannot be too strongly emphasized that experience is required for the colony agglutination. The chief pitfall is nonspecific agglutination, due to the use of too low a dilution of serum, within the range of group agglutination. Some typhoid serums contain these group agglutinins to a greater degree than others, and are, therefore, not so useful for this purpose. All preliminary reports based on colony agglutination should be confirmed by subculture on Russell's double sugar agar, from which the usual confirmatory tests may be carried on.

Our experience with feces cultures for the diagnosis of typhoid has been very satisfactory, with the result that our laboratory now receives more specimens for cultural methods than for agglutination tests, thus indicating that the practitioner will utilize any laboratory procedure that demonstrates results.

The typhoid bacillus is present in the feces from the time of onset of the disease, and in some cases even earlier (precocious carriers).[9] Feces culture, therefore, is a useful diagnostic method, regardless of the stage of the disease. In 108 cases of clinical typhoid fever from which a single specimen of feces was submitted, varying between the third and the forty-eighth day of the attack, we isolated the typhoid bacillus in 86 or 80 per cent.[10] Of those received during the first week, 66 per cent were positive; during the second week, 77 per cent; during the third week, 79 per cent, and after the twenty-first day, 100 per cent of this series yielded positive results.

Paratyphosus A.—Judging from the literature this is relatively rare. Little difficulty is usually encountered in its identification.

Paratyphosus B.—It now seems clear that this term has been applied to two organisms which are quite distinct, bacteriologically and clinically. Jordan's classification into Salmonella schottmülleri, which usually causes prolonged paratyphoid fever, and Salmonella aertryckei, associated with explosive food poisoning, has cleared up a great deal of the existing bewilderment. While this differentiation is not without exception,† the two strains are bacteriologically distinct and should not be confused. Heretofore, many bacteriologists, particularly in Germany, have failed to make this distinction, and there is still a tendency there to consider the *aertrycke* type merely a variety of the species paratyphosus B. The English workers, on the other hand, have complicated the situation by describing many different strains—"Mutton," "Newport," etc.—which are, apparently, practically identical with S. aertryckei.[11] That this confusion is becoming more confounded is indicated by a recent description[12]

*It is interesting to note that the classical textbook description of the appearance of the colony of B. typhosus is that of the rough variant. It has been our experience that this form rarely occurs in freshly isolated cultures; the smooth form predominates, with a regular edge, and smooth, glistening surface. (See Grinnell, F. B.: J. Immunol. **19**: 457, 1930.)

†We have isolated the Schottmüller type from two explosive outbreaks of food poisoning, one due to sausage and the other, ground beef sandwiches.

of an organism, seemingly belonging to the *aertrycke* species, which, because it shows certain serologic differences, has been given the name "Salmonella Eastbourne." It is questionable whether minor antigenic differences warrant the creation of a new species. On this basis it would be just as logical to name each individual strain of S. morgani as a separate species. As Jordan remarks,[11] "Until the range and significance of antigenic variation become more fully understood than they are at present, the multiplication of specific names may well be kept on a conservative basis."

Paratyphosus "C."—It is now generally believed that this strain, found in the Balkans and elsewhere during the war, is identical with B. suipestifer (S. cholerae suis).

S. Morgani No. 1.—Similar to the paratyphoid bacilli in its greater resistance to brilliant green, this organism is more easily isolated from feces than is B. typhosus. As already mentioned,[7, 8] it has been recovered from blood cultures as well as from the feces in cases clinically resembling paratyphoid fever. It has also been reported[13] as the cause of a severe ulcerative colitis. Since it is found, not infrequently, in the feces of normal persons, its significance is doubtful, unless the isolation from the intestinal tract is supported by a positive blood culture or a positive agglutination test with the patient's serum. At any rate, such a finding should not be summarily dismissed as of no importance.

S. Enteritidis.—This organism, together with S. cholerae suis, has been found associated with cases of food poisoning, but they are rarely isolated from human feces. In the study of some 20,000 feces cultures in Alabama we have not once found these bacteria.

The Dysentery Group.—Here, more than with any other intestinal pathogen, is the laboratory confronted with technical difficulties on the one hand, and, on the other, with the necessity of isolating the causative organism as the only sure proof of the cause of the infection. Even more common than for B. typhosus is the occurrence of dysentery agglutinins in normal persons.[14] Cross-agglutination between the different members of the group is a common phenomenon and one must resort to cultural methods to establish the diagnosis. Yet the procedures at our command are notoriously uncertain. Enrichment methods fail because there is no selective medium as in the case of the typhoid-paratyphoid group. The dysentery bacilli are fragile, and are overgrown rapidly, necessitating the use of fresh specimens.

The ideal procedure for collection of the specimen is to hospitalize the patient, collect the specimen with the aid of a proctoscope, and start the cultures at once, at the bedside, if the laboratory is at all remote. The specimen should be carefully examined for flecks of blood and mucus. These should be selected, rinsed in broth or salt solution, and streaked on Endo plates. Lacking these facilities, fair results may be obtained by preserving the feces in 30 per cent glycerin.

The most common member of this group in the United States is the Flexner strain, but in outbreaks of dysentery, variants, either cultural or serologic, may occur[15] and a strain should not be too hastily discarded as of no importance, merely because it does not conform, in all respects, to the type. Here again,

agglutination tests with the patient's serum may be useful in establishing the significance of a questionable culture. According to Koser and his associates,[16] the Sonne type is not uncommon as a cause of dysentery in this country.

Related Bacteria of Doubtful Significance.—Everyone who has had extended experience with fecal bacteriology, has found numerous unclassifiable bacteria which sufficiently resemble the established pathogenic types to cause at least momentary confusion.

Since the first differentiating criterion, upon which all differential media are based, is failure to ferment lactose, naturally any nonlactose-fermenting colony found on the plates should rightly excite suspicion. Too much importance should not be placed on the colony characteristics, such as shape, size, opacity, etc., since colonies of the same strain may vary markedly, particularly if the organism is susceptible to dissociative changes. The culture on Russell's double sugar agar furnishes a basis for the rapid elimination of many of these bacteria which have no significance as disease-producers. In most instances the character of the growth may, at once, eliminate the culture as of no importance, without the necessity for further study. Slide agglutination is a useful rapid method of obtaining preliminary information. The sugar reactions, followed by specific agglutination in known monovalent serum, then establish the characteristics necessary to indicate whether or not further study is warranted.

In spite of these procedures which serve, in most instances, either to identify or eliminate the culture, one not infrequently finds strains which differ from some classified member of the Salmonella group only in their action on a single carbohydrate. We have a collection of some 200 such strains, almost every one differing in some respect from the others. They have been isolated from normal feces as well as from patients with enteric disease. Some resemble paratyphosus B, except in their inability to utilize sorbite. Others fail to ferment rhamnose, and none agglutinates in any paratyphoid B serum which we have used. From time to time, considerable study has been given to these cultures, but their chief bacteriologic importance seems to lie in their confusing resemblance to the known pathogenic Salmonellas, with the consequent necessity for their differentiation in arriving at the etiologic agent in any given case.

CONCLUSIONS

We have tried to show (1) that intestinal bacteriology has an essential place in the diagnostic laboratory and (2) that bacteriologic studies of the feces are of practical importance, from the standpoint of both promptness and accuracy.

Much excellent clinical material which would add to our knowledge of this difficult field of bacteriology undoubtedly goes to waste for lack of opportunity for thorough study. If more of these patients could be made available for bacteriologic examination, not only would the laboratory benefit by the accumulation of information, but clinical medicine woud be improved by more accurate diagnosis in many baffling and obscure conditions.

The methods at our command are by no means perfect, but it has been our experience that, when intelligently and conscientiously used, they compare

favorably with many of our accepted examinations. Their very lack of perfection and our inadequate knowledge are incentives to more extensive, as well as intensive, study. There is a large field for research among the intestinal pathogens, not only in technical methods for their isolation and identification, but also to increase our knowledge of their significance as the causative factors in enteric diseases.

REFERENCES

1. Stitt, E. R.: Practical Bacteriology, Blood Work and Animal Parasitology, ed. 9, P. Blakiston's Son & Co., Philadelphia, p. 176.
2. Jordan, E. O.: J. Infect. Dis. **38**: 306, 1926.
3. Park, Williams and Krumwiede: Pathogenic Microorganisms, ed. 8, 1924, Lea & Febiger, Philadelphia, p. 128.
4. Jordan, E. O., and Harmon, P. H.: Differential Medium for Schottmüller and Aertrycke Strains of Paratyphoid, J. Infect. Dis. **42**: 238, 1928.
5. Simmons, J. S.: J. Infect. Dis. **39**: 209, 1926.
6. Coleman, M. B.: J. Lab. & Clin. Med. **16**: 396, 1931.
7. D'Aunoy, R.: Am. J. M. Sc. **178**: 834, 1929.
8. Havens, L. C., and Mayfield, C. R.: J. Prev. Med. **4**: 179, 1930.
9. Gay, F. P.: Typhoid Fever, 1918, Macmillan Co., New York, p. 121.
10. Havens, L. C., and Dehler, S. A.: J. Prev. Med. **1**: 359, 1927.
11. Jordan, E. O.: J. Prev. Med. **2**: 279, 1928. (An excellent description of the bacteriology and epidemiology of the paratyphoid group.)
12. Leslie, P. H., and Shera, A. G.: A New Serologic Type of Salmonella, J. Path. & Bact. **34**: 533, 1931.
13. Thjötta, T.: J. Infect. Dis. **43**: 349, 1928.
14. Havens, L. C., and Mayfield, C. R.: J. Prev. Med. **5**: 295, 1931. (Ritchie, quoted by Stitt: Practical Bacteriology, Blood Work, Parasitology, ed. 8, Philadelphia, p. 178.)
15. MacKenzie, G. M., and Batt, L. N.: J. Immunol. **19**: 371, 1930.
16. Koser, S. A., Reiter, D. O., Bortniker, E., and Swingle, E. L.: J. Prev. Med. **4**: 477, 1930.

EXAMINATION AND IDENTIFICATION OF PROTOZOA*

By Ernest Carroll Faust, M.A., Ph.D., New Orleans, La.

INTRODUCTION

THE protozoa found in human tissues and exudates include forms belonging to all four major groups of the Phylum Protozoa, namely the Rhizopoda, as illustrated by Endameba histolytica; the Mastigophora, as illustrated by the intestinal flagellates and the trypanosomes; the Sporozoa, as illustrated by the malaria plasmodia, and the Ciliata, as illustrated by Balantidium coli.

Some of these organisms are strictly tissue parasites; others may be regarded as facultative pathogens, and still others are harmless forms of a coprozoic nature living in or passing through the intestinal tract. For purposes of convenience it is desirable to consider these protozoa under two categories, (1) those living in the intestinal tract and adjacent organs and (2) those primarily involving the hematopoietic organs, blood stream and other body tissues.

I. Protozoa of the Intestinal Tract and Adnexa

1. *The amebae (Class Rhizopoda, Order Amoebida).*

The amebae found in the gastrointestinal tract of man include Endameba histolytica, Endameba coli, Endameba gingivalis, Endolimax nana, Iodamoeba bütschlii (vel williamsi), Dientamoeba fragilis and the rare form Caudamoeba sinensis. One of these species (E. gingivalis) is parasitic in the human mouth; the others live in the lower levels of the intestine, being confined almost exclusively to the large bowel. Of these latter E. histolytica and Caudamoeba sinensis are tissue parasites, while the remaining species live free in the lumen of the large bowel and are regarded as nonpathogenic.

TECHNIC FOR EXAMINING INTESTINAL EXUDATES FOR PROTOZOA

In routine laboratory diagnosis I use the following equipment:

1. A good compound microscope, with periplanatic oculars ×6 and ×10; 16 mm., 4 mm. and 1.9 mm. objectives (either achromatic or fluorite), and a mechanical stage.

2. A daylight blue electric lamp, used as a constant source of light.

3. Fecal slides (40 × 75 mm.), of clear white glass, clean of dirt and oil film.

4. Cover glasses (22 mm. square, not over 18 μ in thickness), spotlessly clean.

5. Toothpicks and specimen applicators.

6. A dropping bottle of physiologic saline solution.

7. A dropping bottle of Donaldson's iodine solution (saturated solution of iodine in 5 per cent aqueous potassium iodide).

*From the Parasitology Laboratory, Department of Tropical Medicine, Tulane University Medical School.

The following technic is employed: A fleck of the specimen to be examined is thoroughly mixed on the slide in a drop of physiologic saline solution. This is streaked across two cover glass widths and a cover glass placed over one-half of the film. A small drop of the iodine solution is then mixed with the uncovered portion of the film and covered with a second cover glass. The film is first examined with the 16 mm. objective and any suspicious objects then studied with the 4 mm. lens. On the unstained side active stages (trophozoites) and cysts will be found in their natural hyaline color; on the iodine stained side these organisms will be stained so that the chromatin material stands out in light relief against the yellowish brown cytoplasm. In other words, the picture with the iodine staining is essentially the reverse of that obtained by the iron hematoxylin technic. The iodine also stains the glycogen masses a more or less deep mahogany brown.

A 2 gram portion of each formed or semiformed specimen is also diluted with 20 c.c. of water, strained through gauze and centrifuged. This frequently yields cysts where the undiluted film is negative. At least three separate specimens and preferably six are requested for examination before a diagnosis of "negative" is finally entered. Freshly passed specimens (not over thirty minutes old) are most satisfactory. Specimens containing oil of any kind are practically worthless for examination and one passed after a saline purgative is almost as unsatisfactory.

Occasionally (perhaps in 5 per cent of the cases), it is desirable to check the temporary iodine films with a Schaudinn-fixed iron-hematoxylin-stained preparation. But experience with over 50,000 fecal examinations has shown that the one method is as dependable as the other, and the simplicity of the former leaves much to be said in its favor. However, in case hematoxylin stained preparations are desired, the following technic is recommended:

1. Smear fecal material on slide evenly and not too thick.
2. Place slide in Schaudinn's fluid[1] heated to a temperature of 60° C. Leave in two minutes.
3. Transfer to 70 per cent alcohol, two minutes.
4. Transfer to 70 per cent iodine alcohol, two minutes.
5. Transfer to 70 per cent alcohol, two minutes.
6. Transfer to 50 per cent alcohol, two minutes.
7. Wash in running water two minutes.
8. Transfer to 2 per cent aqueous iron alum solution at 40° C., two minutes.
9. Wash in running water three minutes.
10. Transfer to ½ per cent aqueous hematoxylin, two minutes. (This time may vary according to the strength of the stain.)
11. Wash in water two minutes.
12. Destain in *cold* 2 per cent aqueous iron alum.
13. Wash in running water ten to fifteen minutes.
14. Run through 50 per cent, 70 per cent, 80 per cent, 90 per cent, and 100 per cent alcohol, two minutes each.

[1] Schaudinn's fixing fluid. Prepare the solution as follows: Saturated solution of mercuric chloride in distilled water, 200 c.c.; 95 per cent alcohol, 100 c.c.; glacial acetic acid, 15 c.c. (The acid should not be added until the fluid is to be used.)

15. Xylol two minutes.
16. Mount in balsam or euparal, using a No. 1 cover glass.

ENDAMEBA HISTOLYTICA (Fig. 1 *A*-1 *F*)

This organism is the well-known tissue pathogen. It commonly lives in the wall of the large bowel, particularly the cecum and appendix, but occasionally the adjacent levels of the ileum may be involved. Secondarily this ameba may migrate through the blood stream to the liver, where a focus of infection may develop; more rarely the lungs, brain, and other organs may be parasitized.

In an acute or subacute enteritis, the organism will be passed in the active (trophozoite) stage (Fig. 1 *A*), in a liquid or semiliquid exudate containing many red blood cells, necrotic tissue cells, but relatively few pus cells. In order

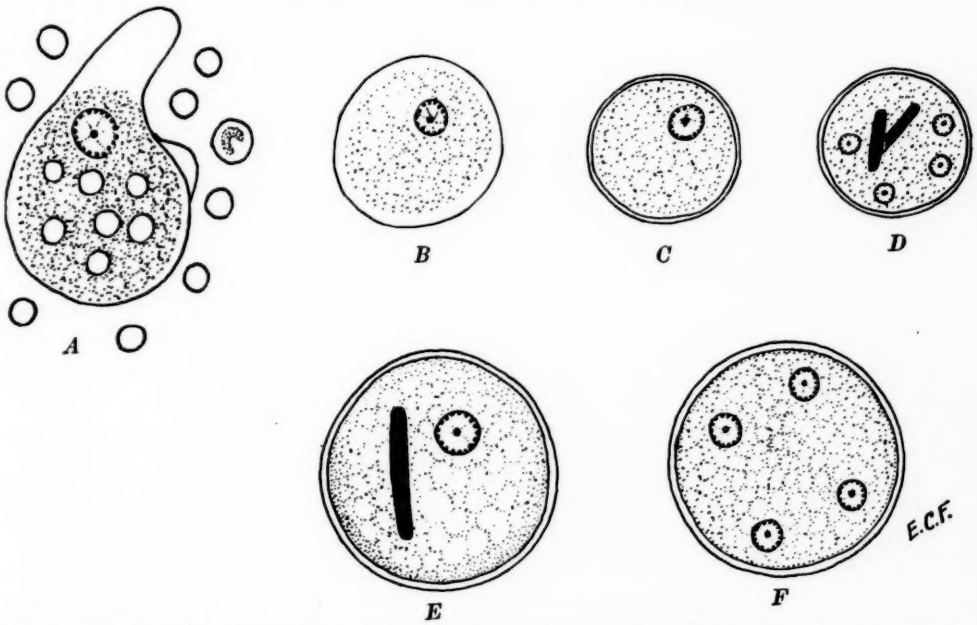

Fig. 1.—Endameba histolytica. *A*, active trophozoite; *B*, precystic stage; *C-F*, small and large races of cysts. *A*, *B*, ×1300; *C-F*, ×2000.

to make a satisfactory diagnosis of such material it is essential that the freshly passed exudate should be examined immediately upon being passed, care being taken that it is not chilled. A fleck of the bloody mucus is placed on a clean microscopic slide, diluted with tepid physiologic saline and mounted with a clean cover glass. These amebae will be seen moving about quite actively in the medium, throwing out their clear pseudopodial processes first in one direction, then in another, with the nucleus near the forward end of the organism. They vary in size from 18 to 40 μ. Frequently they will be found to contain ingested red blood cells and rarely bacteria. While it is at times possible to find this active stage of E. histolytica in the stool for several hours after evacuation, it frequently dies in a half hour or less, particularly in an exudate where putrefaction is going on. In a semidegenerate condition, it may be confused with monocytes, which, however, have much larger nuclei. The large mono-

nuclears in bacillary dysentery exudate are frequently mistaken for E. histolytica. It is a safe principle never to diagnose such a form as an ameba unless definite movement is demonstrated. At times these amebae have degenerated in the lumen of the large bowel and an otherwise typical exudate will show no living organisms. Under such circumstances a high proctoscopic examination may reveal typical crateriform ulcers, from the centers of which living amebae may be obtained. It must be emphasized, however, that only about 37 per cent of amebic lesions occur in the rectum and that the greatest number of primary lesions occur in the cecum and appendix, which cannot be examined with a tube. Cysts of E. histolytica are not passed in a liquid exudate, nor does encystment occur in such a medium after it has been expelled from the bowel. Charcot-Leyden crystals are frequently seen in typical amebic dysentery exudate.

The encysted stage is found in formed stools of both carrier cases and those having an intermittently formed and liquid stool. These cysts are typical (Figs. 1 *C*-1 *F*). They are spherical bodies, 9 to 16 μ in diameter, with a thin cyst membrane, finely granular cytoplasm, and one to four nuclei, depending on their degree of ripeness. These nuclei are characteristic, with a fine chromatin dot (karyosome) in their center suspended in a stellate net of achromatic fibrils, and a peripheral chain of beadlike or plaquelike chromatin particles just within the nuclear membrane. There are no red blood cells or other undigested food particles within the cyst; these have all been expelled just before encystation occurs. However, in about 10 per cent of the cysts one or more sausage-shaped chromatoidal bodies are found in the cytoplasm. These have a chromatin staining reaction and are regarded as excess chromatin extruded from the nucleus at the time of encystation.

In semiformed stools precystic forms of E. histolytica are not infrequently found. These (Fig. 1 *B*) are uninucleate rounded-up specimens, from which the undigested food has been or is about to be extruded, but as yet without a cyst wall. If the stool is allowed to desiccate they will frequently encyst.

In examining for E. histolytica in amebic hepatitis it should be remembered that the organism is not commonly found in the material aspirated from the "abscess" cavity, but lives in the wall of the pocket and can be aspirated out after the pocket has been drained. Only trophozoites occur in this location.

Some workers prefer to place a portion of each sample suspected of harboring E. histolytica in a culture medium and incubated at 37° C. for twenty-four to forty-eight hours. Probably the most satisfactory medium is that of Boeck-Drbohlav (1925), consisting of a slanting egg substratum and a supernatant layer of Locke's solution (serum-egg albumin). The amebae grow best along the surface of the solid substratum. However, this technic can never take the place of the routine examination of gross and concentrated samples.

Craig (1927) has introduced the use of a complement fixation test both as a routine and a check for microscopic examination for E. histolytica. This is a valid method in so far as there is a systemic reaction in the patient harboring the organism.

E. histolytica may be expected in 5 or more per cent (up to 50 per cent) of the population of any given area in the United States, the amount of the infection depending on both latitude and local sanitary conditions.

CAUDAMOEBA SINENSIS (Fig. 2 *A*-2 *B*)

This organism has been found in four cases of acute amebic enteritis in native patients in Peking, China (Faust, 1923). Only the trophozoite stage has been seen. It is distinguished by having an almost constant flowing movement from the forward end of the organism, and at the opposite end a small but distinct caudal process. The nucleus has a stellate karyosome and lacks the distinct peripheral chromatin beading of E. histolytica.

ENDAMOEBA GINGIVALIS (Fig. 3)

This organism lives in suppurative pockets in the gums, particularly in persons having pyorrhea. Scrapings of the gums around carious teeth fre-

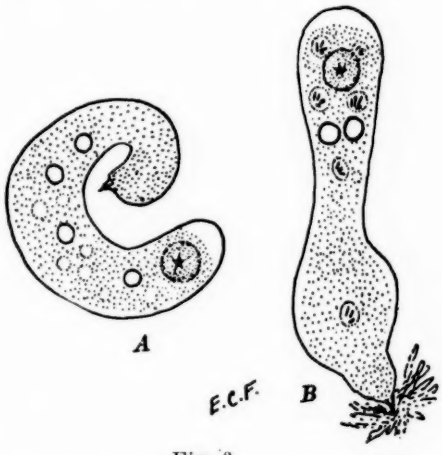

Fig. 2

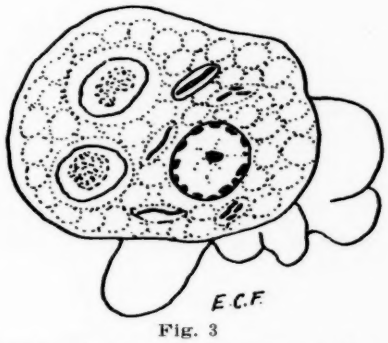

Fig. 3

Fig. 2.—Caudamoeba sinensis. Trophozoites, showing characteristic structure and movement. ×1300.
Fig. 3.—The active Endameba gingivalis. ×2000.

quently yield this organism, which is fairly active at body temperature. The pseudopodia are limpid clear but small; there is a tendency for several to be extruded at one time. These amebae range in size from 10 to 40 μ, and have a narrow marginal ectoplasm and a highly vacuolated granular endoplasm. The nucleus has a karyosome, usually central in position and a ring of chromatin granules just within its membrane. Food vacuoles contain bacteria and white cell casts, possibly also red blood cells. Although the organism probably has an encysted stage it is doubtful if the cyst has ever been observed.

ENDAMOEBA COLI (Fig. 4 *A*-4 *E*)

Endamoeba coli is the most common protozoan found in the stool. This organism may be seen in the trophozoite stage in diarrheic stools or after a saline purgation (Fig. 4 *A*). It varies in size from 15 to 50 μ. The cytoplasm is grayish-green and is very granular. The movement is sluggish, and little difference can be found between ectoplasm and endoplasm. Usually there are several food vacuoles, containing bacteria and vegetable cells. The nucleus is large and is readily seen in unstained specimens. The karyosome is either a single excentric mass or is composed of a clump of several chromatin granules. There is a peripheral band of coarse chromatin granules. In iodine-stained films the trophozoite frequently contracts into a dense, almost opaque mass.

The cyst (Fig. 4 *B*-4 *E*) is spherical or subspherical, varies in size from 10 to 33.5 μ, has a relatively thick capsule and a dense cytoplasm, in the midst of which can be seen the distinct nuclei (1 to 8 or more, depending on their stage of ripeness). These nuclei are best observed in iodine-stained films. If five or more nuclei can be counted (Fig. 4 *D*-4 *E*), there is reasonable certainty that the organism is Endamoeba coli, although rarely up to 8 nuclei have been found in E. histolytica cysts. In unripened cysts with 1 to 4 nuclei, it is necessary to determine the nuclear structure. Occasionally chromatoidal bodies occur; these (Fig. 4 *D*) are falciform or splintered structures, very different from the chromatoidals of E. histolytica cysts. From time to time E. coli cysts show

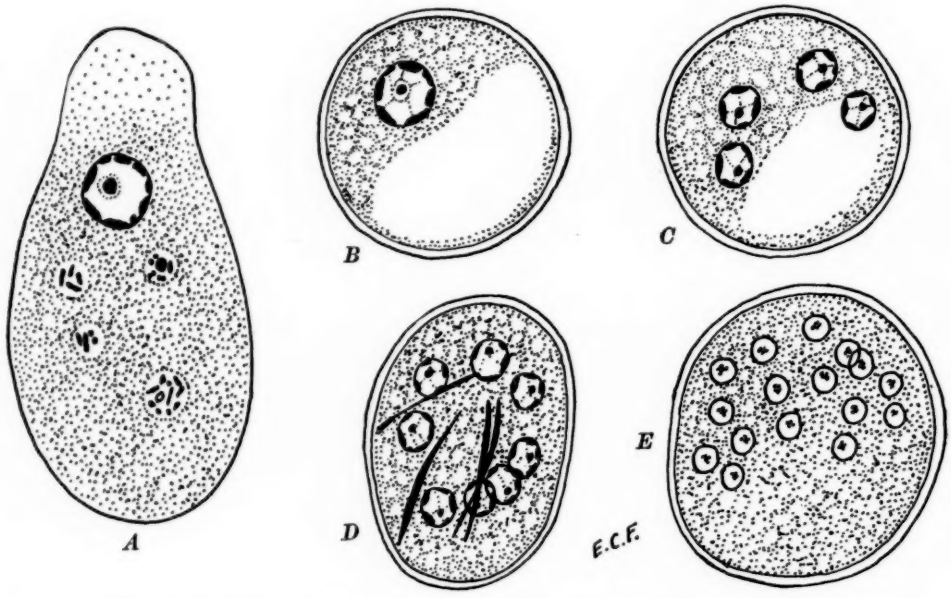

Fig. 4.—Endameba coli. *A*, trophozoite; *B-E*, representative cysts. ×2000.

dense diffuse glycogen masses (Fig. 4 *B*-4 *C*), which obscure the other structures in the iodine stained preparation. The Councilmania lafleuri of Kofoid and Swezy (1923) is regarded by most workers as an aberrant form of E. coli.

ENDOLIMAX NANA (Fig. 5 *A*-5 *D*)

This little ameba is a common inhabitant of the intestinal tract. In diarrheic or semiformed stools, it may be seen in the trophozoite stage, with granular vacuolated endoplasm and clear limpid ectoplasm, the latter frequently thrown out as hyaline pseudopods (Fig. 5 *A*-5 *B*). The nucleus of the motile stage is not usually seen in unstained specimens. The cyst (Fig. 5 *C*-5 *D*) is oval or less commonly rounded (6-10 μ), has a distinct wall, a distinctly vacuolated cytoplasm and 1 to 4 nuclei, which look like punched out holes with a laterally disposed chromatin clump. The chromatoidal masses in the cytoplasm are small and frequently crescentic. Diffuse glycogen masses in the cytoplasm are not uncommonly seen (Fig. 5 *D*).

IODAMOEBA BÜTSCHLII (Fig. 6 *A*-6 *D*)

This species is never as commonly encountered as E. histolytica, E. coli and Endolimax nana. It is most usually observed in formed stools. The trophozoite (Fig. 6 *A*) varies in size from 5 to 20 μ, is sluggish, the cytoplasm is finely granular, and the nucleus, which is seen with difficulty in unstained specimens, has a large chromatin blob surrounded by a more or less stellate frame of "peripheral chromatin." The cysts (Fig. 6 *B*-6 *D*) are frequently referred to as "iodine

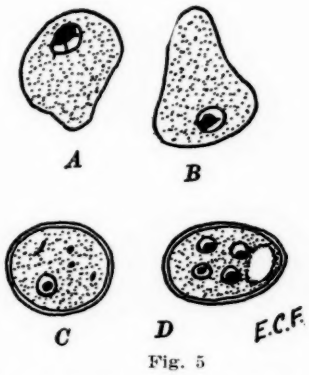

Fig. 5

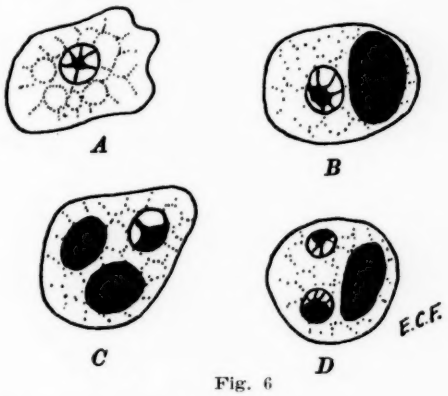

Fig. 6

Fig. 5.—Endolimax nana. *A*, *B*, trophozoites; *C*, *D*, cysts. ×2000.
Fig. 6.—Iodamoeba bütschlii. *A*, trophozoite; *B-D*, cysts. ×2000.

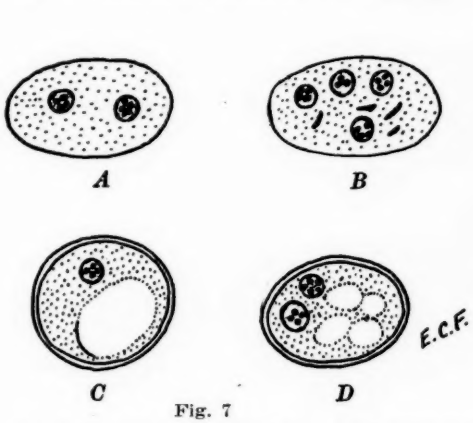

Fig. 7

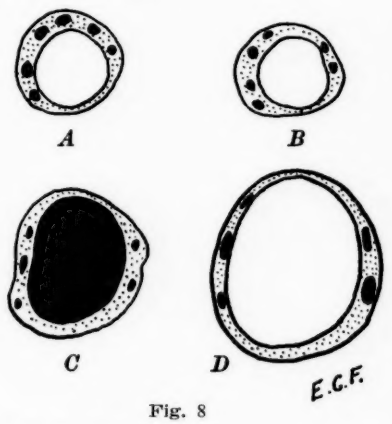

Fig. 8

Fig. 7.—Dientamoeba fragilis. *A*, *B*, trophozoites; *C*, *D*, cysts. ×2000.
Fig. 8.—Representative specimens of Blastocystis hominis from human feces. ×2000.

cysts," because of the large definitely delimited glycogen mass which stains a mahogany brown in the iodine treated preparation. They are irregularly oval or rounded, vary in size from 6 to 17 μ and have a distinct capsule. There is usually only one nucleus, which is similar to that of the trophozoite stage. The cytoplasm has one or more vacuoles but no chromatoidals have been described.

DIENTAMOEBA FRAGILIS (Fig. 7 *A*-7 *D*)

This rare ameba is usually found to have two nuclei in the trophozoite stage (Fig. 7 *A*-7 *D*), although uninucleate and quadrinucleate active forms have been observed. The organism ranges from 3.5 to 12 μ in size, is actively motile

and has clear lobate or sinusate pseudopodia. The endoplasm is granular and contains food vacuoles. The structure of the nucleus is characteristic, with a delicate membrane, and a collection of several distinct karyosomal granules near the center. Uninucleate and binucleate cysts (Fig. 7 *C*-7 *D*) have been observed by Kofoid, but the organism is usually so delicate that it degenerates rapidly in passed stools.

Councilmania tenuis, C. dissimilis and Karyamoebina falcata, forms described by Kofoid and his colleagues (1924, 1928) apparently have not been observed by other workers.

BLASTOCYSTIS HOMINIS (Fig. 8 *A*-8 *D*)

Although this yeastlike organism is in no wise related to the amebae, it is frequently confused with them by the unsuspecting diagnostician. These organisms are spherical, colorless, highly refractile and measure from 5 to 30 μ in diameter, with a considerable size variation in any particular specimen of stool. They consist of a vacuolated center surrounded by a thin peripheral rim of cytoplasm with one or more nuclei. The body is enclosed in a relatively impermeable capsule. At times dividing dumbbell forms are seen. These organisms frequently shrink in the iodine-stained films.

Blastocystis is most commonly found during the season when fresh fruits such as grapes, apples and pears, are eaten raw. This organism is a most serious contaminator of E. histolytica cultures.

2. *Flagellates (Class Mastigophora).*

The flagellate protozoa found in the gastrointestinal tract of man include Trichomonas hominis, Trichomonas buccalis, Chilomastix mesnili, Giardia lamblia, Embadomonas intestinalis, Embadomonas sinensis, Enteromonas hominis, Tricercomonas intestinalis, Cercomonas longicauda and Bodo caudatus. Of these species Trichomonas hominis, Chilomastix mesnili and Giardia lamblia are the common intestinal forms, Trichomonas buccalis lives in the mouth, and the other species are without doubt coprophagous nonpathogenic organisms seen from time to time in diarrheic stools. In addition, Trichomonas vaginalis occurs in a fairly high percentage of specimens of urine from female patients and is occasionally found in catheterized urine of male patients.

TRICHOMONAS HOMINIS (Fig. 9)

This flagellate is a comparatively common parasite, growing for the most part in the upper levels of the large bowel. It is most commonly observed in diarrheic or semiformed stools and is apparently associated with low gastric acidity, which permits passage of the trophozoite stage through the stomach without injury to the parasite. The organism does not have a cystic stage. The active organism is more or less heart-shaped, with a length varying from 7 to 20 μ and a breath of 3 to 7 μ. Its marked motility is due to the combined action of its flagella, undulating membrane and plastic body. Forms have been described with three anterior flagella (Tritrichomonas), four anterior flagella (Tetratrichomonas) and five anterior flagella (Pentatrichomonas ardin delteili). Behind the base of these flagella on the ventral side is a slitlike buccal cavity (cystostome). Arising from the point of insertion of the flagella and descend-

ing along the dorsal side in a slightly spiral course, is the undulating membrane which ends near the posterior end of the body. Attached to its margin is an accessory flagellum, which at times has a free trailing posterior end, while along its inner margin is an axoneme. Arising just behind the flagellar origin and continuing through the body distalwards to break through the posterior end is the semirigid spikelike axostyle. An oval nucleus may be seen near the anterior end of the organism. The cytoplasm is finely granular and contains numerous vacuoles.

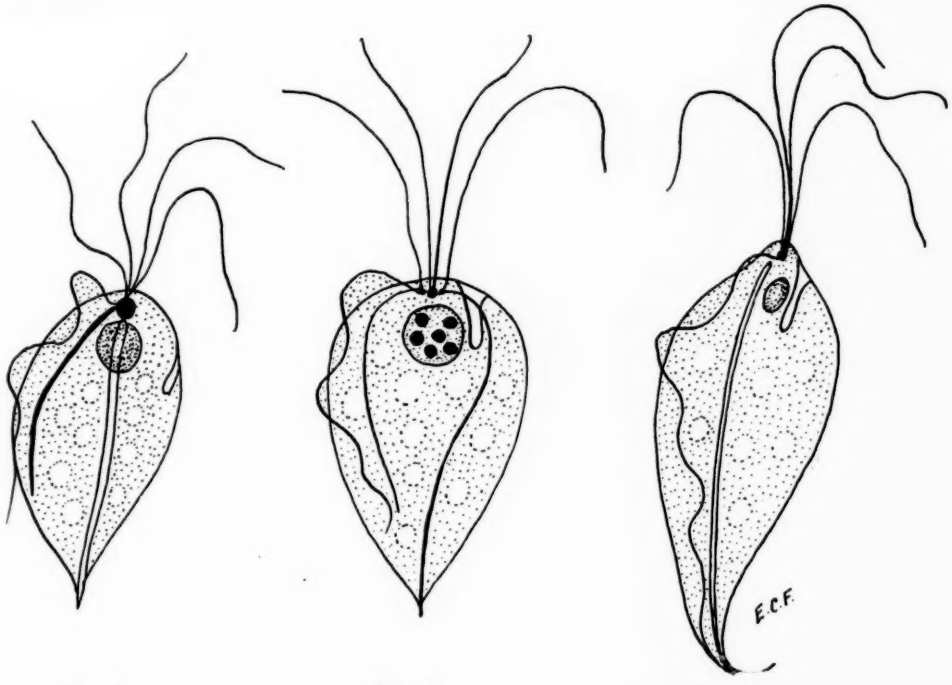

Fig. 9 Fig. 10 Fig. 11

Fig. 9.—Trichomonas hominis. ×2000.
Fig. 10.—Trichomonas buccalis. ×2000. (Adapted from Kofoid.)
Fig. 11.—Trichomonas vaginalis. ×2000. (Adapted from Kunstler.)

Trichomonas hominis moves with a nervous jerky, somewhat corkscrew forward motion. The undulating membrane, which can be seen best when the animalcule is more or less quiescent, is diagnostic for the genus. Multiplication is by longitudinal binary fission.

TRICHOMONAS BUCCALIS (Fig. 10)

This organism (7-12 μ by 4-8 μ) lives and breeds in the tartar around carious teeth and in pus pockets in cases of pyorrhea. It has four anterior flagella and a delicate axostyle which barely protrudes beyond the posterior end of the body. The nucleus is a large oval body. The undulating membrane has a decided twist near the midbody level.

TRICHOMONAS VAGINALIS (Fig. 11)

This large flagellate species is found in vaginal smears and catheterized urine of a fairly high percentage of women and is occasionally found in catheter-

ized urine of the male subject. It measures 15 to 25 μ in length by 10 to 15 μ in breadth. There are four flagella arising from the slightly protuberant anterior end. The undulating membrane spirals to the posterior end of the animalcule but has no free trailing flagellar termination. The weakly developed axostyle likewise has no free termination. The distal end of the organism is acutely pointed and often curved.

CHILOMASTIX MESNILI (Fig. 12 *A*-12 *B*)

This flagellate organism is found in both diarrheic and solid feces. In the former medium it is usually in the trophozoite stage, although when the material

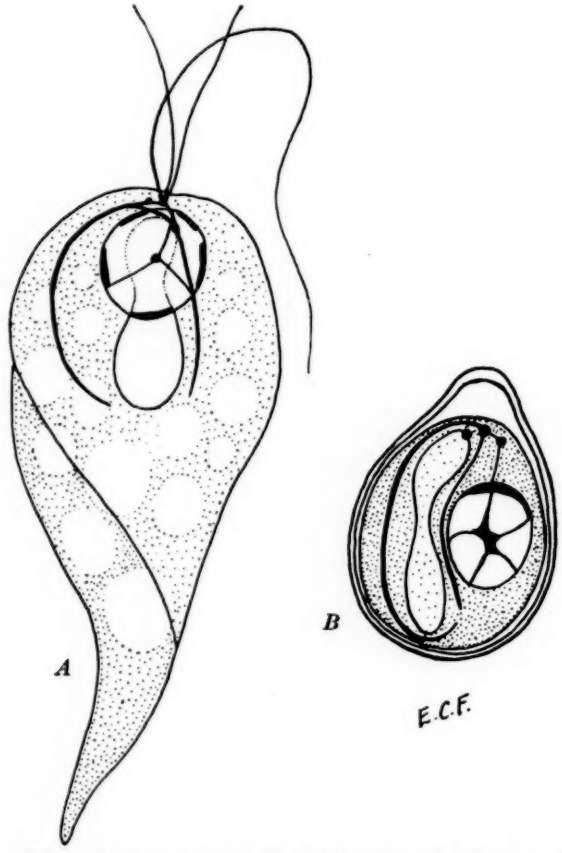

Fig. 12.—Chilomastix mesnili. Left, trophozoite; right, cyst. ×4000.

is centrifuged encystment frequently results from the mechanical agitation; in the formed specimen cysts are the usual stage observed. The motile form (Fig. 12 *A*) varies from 6 to 24 μ in length and 2 to 9 μ in transsection. The anterior end is oval and the posterior end is drawn out into a plastic caudal process. There is a pronounced counterclockwise torsion to the body. The conspicuous cytostome or mouth begins at the anterior end on the ventral aspect and extends backward as a dumbbell-shaped cleft of the cytoplasm toward the midplane of the body. Projecting from the anterior end are three delicate flagella. The large oval or subspherical nucleus can rarely be seen without hematoxylin staining.

The cytoplasm is hyaline, finely granular and contains several vacuoles. The organism exhibits an active, jerky, spiral movement, during which the caudal process may elongate considerably. Reproduction is by longitudinal fission.

The encysted stage (Fig. 12 *B*) is characteristically lemon-shaped. It measures from 6.5 to 9 μ in length by 4.5 to 6 μ in breadth. Iodine-stained specimens

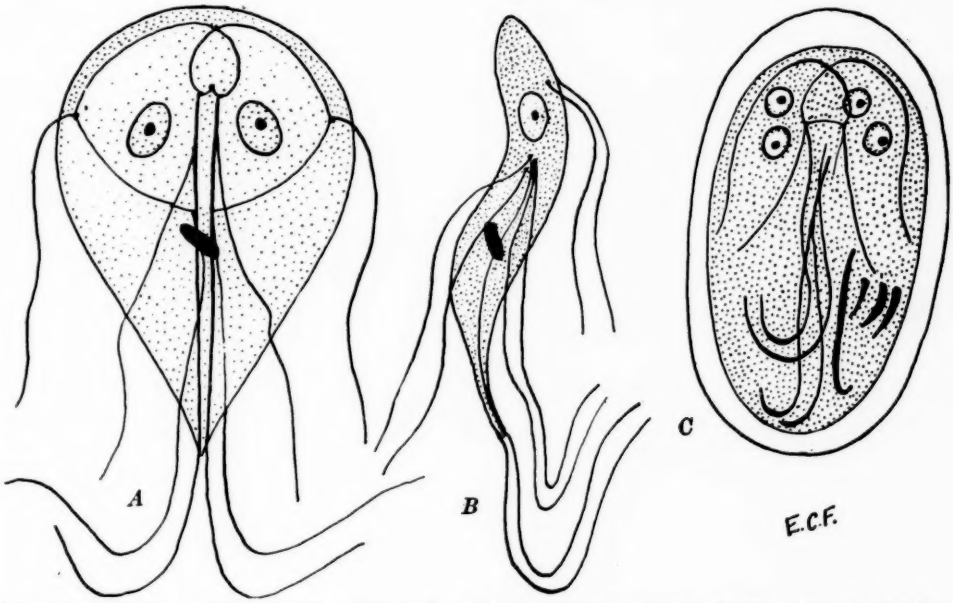

Fig. 13.—Giardia lamblia. Left, ventral view of trophozoite; center, lateral view of trophozoite; right, cyst. ×6000.

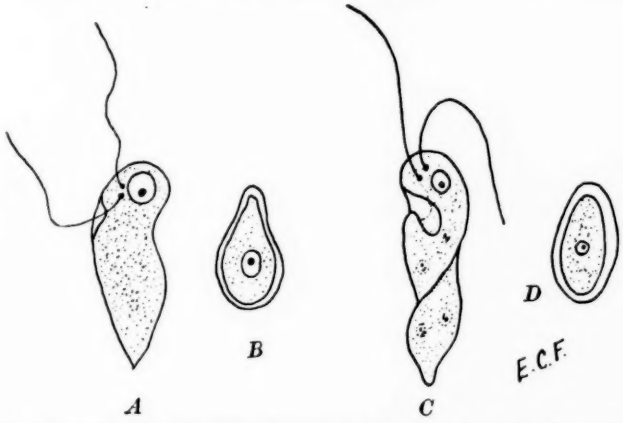

Fig. 14.—Embadomonas spp. *A*, trophozoite and *B*, cyst of E. intestinalis; *C*, trophozoite and *D*, cyst of E. sinensis. ×2500.

show evidence of the cytostome and retracted flagella, as well as a definitely outlined enveloping cystic capsule.

GIARDIA LAMBLIA (Fig. 13 *A*-13 *C*)

In its trophozoite stage (Fig. 13 *A* and 13 *B*), which is found only in unformed feces, this flagellate exhibits a very rapid movement, produced by the 4 pairs of flagella arising from the ventral side of the body. The organism is

top-shaped in contour, with a rounded anterior and a pointed posterior end. Its dorsal side is rounded and its ventral aspect cupped, so that on lateral view (Fig. 13 *B*) it looks something like a half pear which has been cored. The size ranges from 9 to 20 μ (length) by 5 to 12 μ (breadth). The cytoplasm is finely granular and two nuclei can usually be seen.

The cyst (Fig. 13 *C*), which is the stage of the organism most usually seen, is oval, measuring 8 to 14 μ long by 6 to 10 μ broad. The cytoplasm is definitely

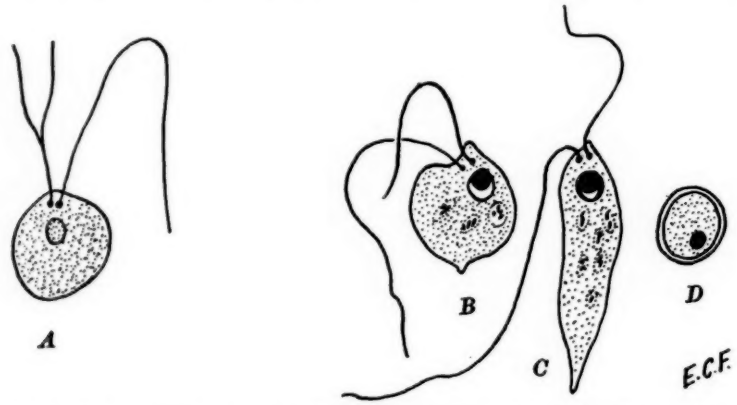

Fig. 15.—*A*, Enteromonas hominis; *B*, *C*, trophozoites of Bodo caudatus; *D*, cyst of B. caudatus. ×2000.

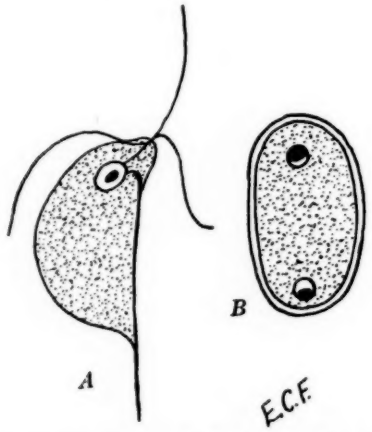

Fig. 16.—Tricercomonas hominis. Left, trophozoite; right, cyst. ×2000. (After Wenyon and O'Connor.)

separated from the enveloping thin-walled hyaline capsule. Usually four spherical nuclei can be observed as well as the retracted flagella. There are also short thick fibrils, which may be associated with the process of reproduction. These cysts have a low specific gravity and are not readily concentrated by centrifuge methods. They stain acceptably with the Donaldson's iodine technic.

EMBADOMONAS (WASKIA) INTESTINALIS AND E. SINENSIS (Fig. 14 *A*-14 *D*)

These minute organisms (4.5-6 μ × 2.5-4 μ) are uncommon compared with Trichomonas, Chilomastix, and Giardia. The trophozoite stage (Fig. 14 *A*, 14 *C*) is irregularly ovate, biflagellate and has a slitlike cytostome. One flagellum

frequently trails backward across the cytostome. The cyst (Fig. 14 *B*, 14 *D*) is pyriform (4.5-6 $\mu \times 3\ \mu$) and easily confused with yeast cells. These coprophagous species are most commonly found in diarrheic stools of persons having a gastrointestinal upset of a chronic or acute nature. I have seen E. intestinalis once in catheterized urine from a female patient.

ENTEROMONAS HOMINIS AND BODO CAUDATUS (Fig. 15 *A*-15 *D*)

These minute subspherical or oval biflagellate species occur in diarrheic stools of persons having a digestive upset. Their minute cysts are easily confused with yeasts. These are not uncommonly seen in contaminated stools.

TRICERCOMONAS HOMINIS (Fig. 16 *A*-16 *B*)

The trophozoite of this small flagellate (4-8 μ in transverse diameter) has 3 anteriorly directed and one posteriorly trailing flagella. The organism is ir-

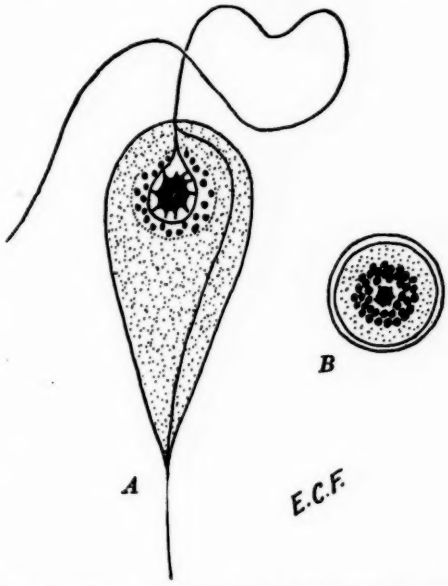

Fig. 17.—Cercomonas longicauda. Left, trophozoite; right, cyst. ×2000. (After Wenyon.)

regularly oval, at times with a suggestion of a caudal projection. The cyst (4-8 μ) is oval and usually binucleate. This organism occurs occasionally in diarrheic stools.

CERCOMONAS LONGICAUDA (Fig. 17 *A*-17 *B*)

The trophozoite of this small flagellate (5-16 μ) is pyriform, has a single very long anteriorly directed flagellum and a posterior one which extends considerably beyond the posterior end of the body. There are numerous granules surrounding the anteriorly disposed nucleus. The cyst (4-7 μ) is subspherical and shows the granules around the nucleus. This organism is found occasionally in diarrheic stools.

3. *The coccidia (Class Sporozoa).*

Only one species of this group has been definitely proved to be a human intestinal parasite. This is Isospora hominis.

ISOSPORA HOMINIS (Fig. 18 *A*-18 *B*)

The organism lives in the villi of the small intestine of man, where schizogony and the sexual products develop and fertilization takes place. The presence of the parasite produces a diarrhea, which persists until the parasites are spontaneously evacuated. The stage recovered from the stool is the oocyst. These are heavily walled hyaline bodies, measuring 25 to 33 μ in length by 12 to 16 μ in cross section. They are irregularly elongate oval in shape and have a delicate capsular pore at one end. The capsule is very resistent to stains. The immature cyst (Fig. 18 *A*) has a single blastomere. In older stools this divides into two, and four internal spores of a curved sausage shape are formed from each of these (Fig. 18 *B*). In the five cases which I have personally seen, the cysts disappeared from the stools in two or three weeks after the onset of the diarrhea, in other words, as soon as the infection spontaneously cleared up.

4. *Ciliates (Class Ciliata).*

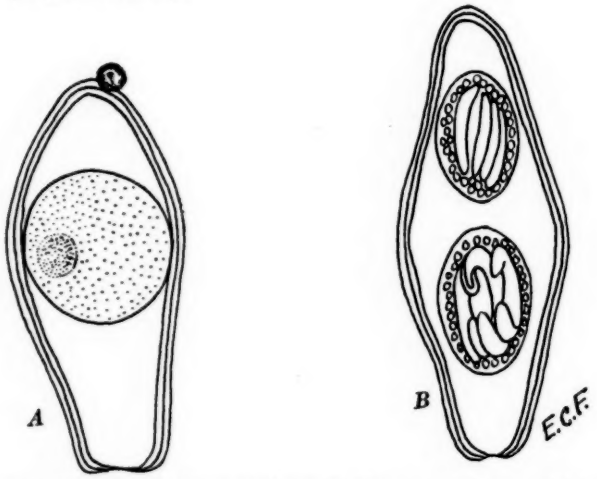

Fig. 18.—Isospora hominis. Left, cyst from fleshly passed feces; right, mature cyst from cultured feces. ×2000.

Only one form of importance is found parasitic in man. This is Balantidium coli from the large bowel.

BALANTIDIUM COLI (Fig. 19 *A*-19 *B*)

This large infusorian form is associated with a pathologic process paralleling that of E. histolytica in the cecum, appendix, colon, and rectum. It is found in man most commonly in the tropics where monkeys are uniformly infected. In temperate zones the pig is an important reservoir of the infection. The trophozoite (Fig. 19 *A*) is oval, slightly attenuated at the anterior end and measures from 30 to 120 μ in length by 20 to 50 μ in greater transsection. An anterolateral invagination leads into a distinct cytostome or mouth cavity. The entire body is covered with many cilia, arranged in spiralled rows. These cilia are the locomotor organs. Internally there is a large kidney shaped trophonucleus and a small kinetonucleus which lies in the hilum of the trophonucleus. There are also food vacuoles containing ingested starch and other food particles. The cyst (Fig. 19 *B*) is spherical, has a well-defined capsule and has a diameter about

that of the greater breadth of the trophozoite. Trophozoites are most common in unformed specimens. Care should be taken not to confuse this organism with numerous infusoria, which get into fecal specimens from contamination.

II. Protozoa of the Hematopoietic Organs, Blood Stream and Other Body Tissues

1. *The hemoflagellates (Class Mastigophora, Family Trypanosomidae).*

This group includes the three species of Leishmania, L. donovani, L. tropica, and L. americana, and the three human trypanosomes, Trypanosoma gambense, T. rhodesiense, and T. cruzi. These forms are usually diagnosed from thin blood or pulp smears, dried and stained with Wright's or Giemsa's stain.

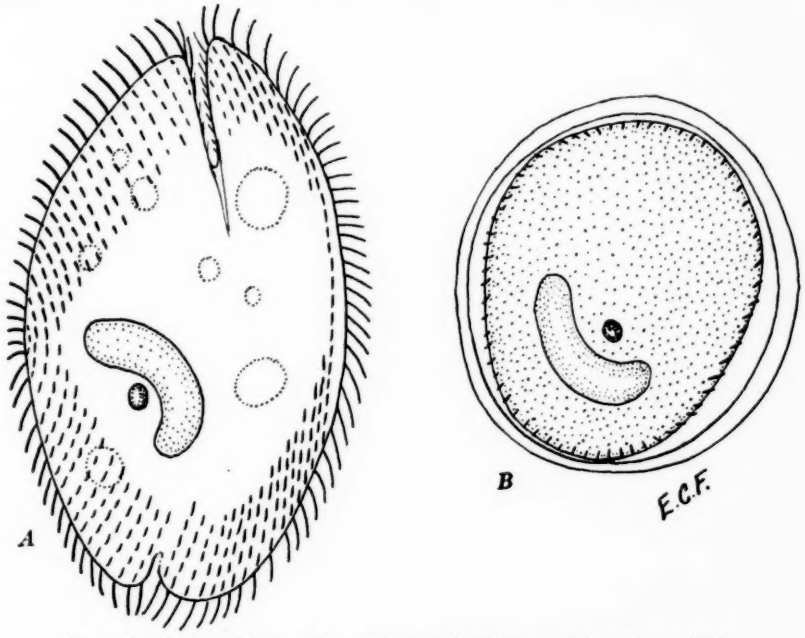

Fig. 19.—Balantidium coli. Left, trophozoite; right, cyst. ×1000.

Leishmania donovani (Fig. 20 *A*-20 *F*)

This species is the causative organism of kala azar, a visceral leishmaniasis producing a hyperplasia of the lymphoid tissue of the hematopoietic organ and the reticuloendothelial system. In the human body the organism is a small oval or torpedo-shaped body parasitic in the cytoplasm of the endothelial monocytes and wandering cells (Fig. 20 *A*), and is occasionally found free in the circulating blood. It measures 2 to 5 μ in length by 1.5 to 2.5 μ in transverse diameter. The cytoplasm stains a delicate blue tint, the trophonucleus, a delicate lavender or violet, and the rod shaped blepharoplast, which lies at right angles to the trophonucleus, takes a dark reddish violet or madder hue. The most ready diagnosis is made from splenic or hepatic pulp smears, although biopsy of subcutaneous tissue may locate the parasites. In smearing tissue pulp the large heavily parasitized monocytes frequently rupture, and the minute parasite will be found spread out in a fan shaped area on one side (Fig. 20 *B*). Frequently only the

trophonucleus and the blepharoplast are well stained. Blood films alone are much less likely to have the organisms. Since kala azar is seldom an acute infection some diagnosticians prefer not to risk spleen or liver puncture and depend on culture of the organism from the monocyte layer of centrifuged blood cells, the culture being made on N.N.N. media, which is incubated at about 20-

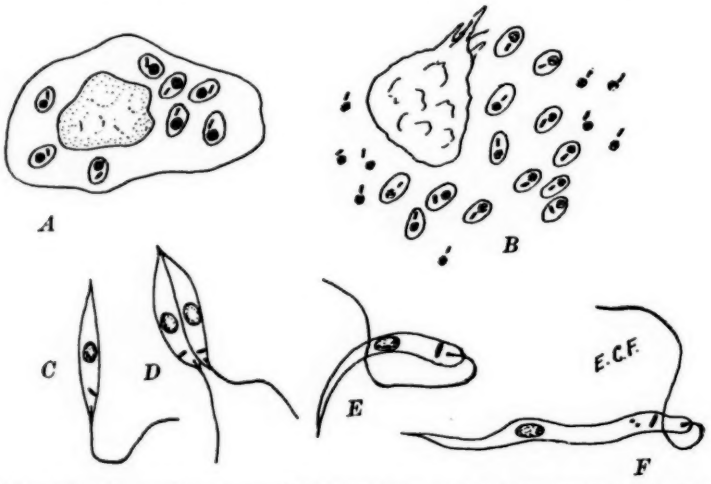

Fig. 20.—Leishmania donovani. *A, B,* leishmaniform stage from endothelial cells of spleen; *C-F,* flagellate stage from culture. ×2000.

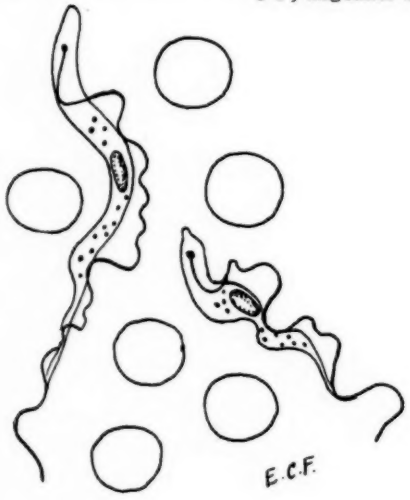

Fig. 21

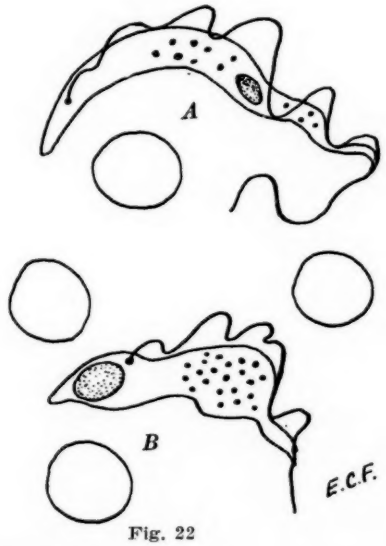

Fig. 22

Fig. 21.—Trypanosoma gambiense in peripheral blood film. ×2000.
Fig. 22.—Trypanosoma rhodesiense in peripheral blood film. *A,* usual form; *B,* posterior nucleate type from rodent host. ×2000.

22° C. for three days or more. The flagellate stage of the organism (Figs. 20 *C*-20 *F*) is found in the water of condensation of the culture (Young and Van Sant, 1923).

Due to an excess of serum euglobulin in the blood plasma of kala azar patients the addition of distilled water to a blood sample (20 c.mm. of blood,

0.6 c.c. aq. dist.) results in a flocculent precipitate in positive cases (Sia, 1921). There is also a positive aldehyde test when 1 c.c. of blood serum of a patient is treated with a drop of strong commercial formaldehyde (Napier, 1922). These tests are specific except in areas where Schistosoma infections are also prevalent. A marked leucopenia with a relative lymphocytosis is a common blood picture in kala azar.

LEISHMANIA TROPICA

This organism, which is structurally similar to L. donovani, produces Oriental sore (Aleppo button, Delhi boil), a cutaneous infection, in which the organisms are found in endothelial cells, large monocytes and ocassionally in polymorphonuclear leucocytes. Smears of the infected tissue properly stained, show these parasites in much the same way as splenic pulp smears reveal L. donovani forms. The organism may be cultured on N.N.N. media.

LEISHMANIA AMERICANA

This organism, which is structurally similar to L. donovani, produces a mucocutaneous infection. The organism can be recovered from the lesions in a similar manner to those of the two preceding species.

TRYPANOSOMA GAMBIENSE (Fig. 21)

This hemoflagellate form is the causative organism of West African or Gambian sleeping sickness, which is characterized primarily by a hyperplasia of lymph gland tissue. It can be recovered from the peripheral blood, enlarged lymph gland pulp or in later cases from sedimented spinal fluid of an infected patient. The parasite in this stage is typically trypaniform, is roughly spindle-shaped, with a narrowly pointed nonflagellar end and an attenuate flagellate end. Spiraling along one side is the undulating membrane, on the margin of which the main portion of the flagellum is attached. The organism measures 16 to 30 μ in length by 1.5 to 2.5 μ in breadth. Near the middle of the body is the large oval trophonucleus, while the flagellum arises from a basal granule, very near the blepharoplast, at the nonflagellar end of the body. The organism divides by longitudinal fission. An intermediate stage occurs in tsetse flies, which serve as transmitting agents. White rats and mice serve as good laboratory animals for the infection. In infected animals a trypanolytic substance is developed which is specific for T. gambiense. This criterion serves to differentiate this species from T. rhodesiense. The organism can be cultured on N.N.N. media. In infected individuals there is a marked anemia and a relative lymphocytosis.

TRYPANOSOMA RHODESIENSE (Fig. 22)

This organism is the causative organism of Rhodesian sleeping sickness. In man it is practically impossible to distinguish this species from T. gambiense. In laboratory animals (rat, guinea pig, rabbit) and rarely in man, there are, in addition to the usual forms, posterior nuclear forms (Fig. 22 *B*) which serve to differentiate the species. This infection is much more rapidly fatal in laboratory animals as well as in man than is T. gambiense infection. Specific trypanolytic and agglutinin substances are also elaborated in this disease.

TRYPANOSOMA CRUZI (Fig. 23)

This organism is the causative organism of Chagas disease or South American trypanosomiasis. The organism in peripheral blood (Fig. 23 *A*) has the typical trypaniform characteristics; it is, however, dimorphic, one form being long and slender, with an elongate trophonucleus and one being shorter, with an oval or nearly spherical nucleus. The flagellum, both in its attached and free portions, is much coarser than that of the African species of man. The parasite does not undergo longitudinal division in the peripheral blood of man. In the tissue, including the heart muscle, lymph glands and brain, T. cruzi assumes a leishmaniform (Fig. 23 *B*) as well as a trypaniform type. There is no marked anemia in the disease although a leucocytosis is characteristic. Laboratory animals can be infected with some difficulty. Transmission is produced by bites of Triatoma megista and related species of bugs.

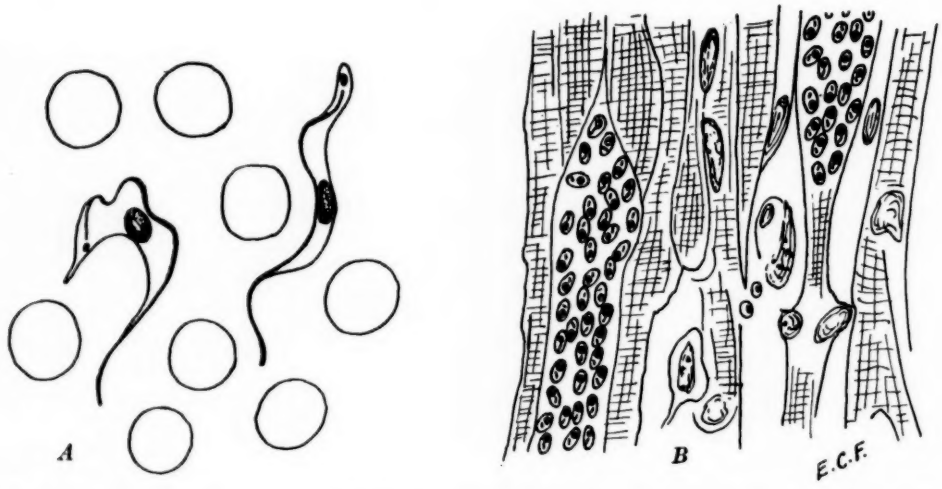

Fig. 23.—Trypanosoma cruzi. *A*, flagellate stage in peripheral blood; *B*, leishmaniform stage in heart muscle. ×2000. (Adapted from Chagas.)

2. *The malarial parasites (Class Sporozoa, Order Hemosporidia, Family Plasmodidae).*

Three species of this group are parasitic in man, Plasmodium vivax (the organism of tertian malaria), P. malariae (the organism of quartan malaria) and P. falciparum (the organism of estivo-autumnal or malignant malaria). The asexual phase develops in man and the sexual phase in the appropriate female anopheline mosquito. It is important not only to differentiate the three species from one another but also to distinguish the several stages of development of each species in the blood.

DIFFERENTIAL CHARACTERISTICS OF THE MALARIAL PARASITES

The usual method of examining blood films for the malaria parasites, as for other infections of the blood stream, is by *the thin blood film.* Practically all of the preparations of socalled typical malaria parasites are those seen in the

thin film. It seems appropriate, therefore, to take up the various stages of the three species of malaria parasites from this point of view.

In each one of the three species, the earliest stage which will be found in examination of the thin blood film is the young growing stage or early trophozoite. In all three species, it is a small rounded body staining azure blue with Wright's or other Romanowsky stains, and having on one side a small brick-red chromatin or nuclear dot.

The Tertian Parasite (Plasmodium vivax).—In the tertian trophozoite (Plasmodium vivax) growth ordinarily takes place from the side opposite the nuclear dot, either as a dense cytoplasmic mass or with the development of plasmodial processes extending into the red blood cell. This growth continues until the parasite has almost completely filled the red blood cell. Meanwhile the cell has become enlarged and pale, and a considerable amount of hematin pigment has accumulated inside of the parasite. Likewise, in the red blood corpuscle outside of the parasite small orange colored dots, known as Schüffner's dots, are usually elaborated. When this trophozoite has increased to its full extent, so that it has almost completely filled the enlarged pale red blood cell, the nucleus divides (process of schizogony) into two, then into four, and subsequently into a larger number of units, so that eventually there will be as many as sixteen to twenty-four of these nuclei that are more or less symmetrically arranged around a residual center containing hematin pigment. This ripe schizont is frequently spoken of as the rosette stage. The red blood cell soon bursts and individual units (merozoites) erupt into the blood stream, each one in turn infecting another red blood corpuscle. This asexual multiplicative process takes place time and again during the incubation or prepatent period, and also to a certain extent after symptoms develop.

As soon as the system of the parasitized individual begins to elaborate antibodies, the malarial parasite, in order to protect itself, also develops gametocytes from some of the trophozoites. These have a more regular outline, being more or less oval. The nuclear dot is somewhat less distinct than it is in the ordinary trophozoite stage, and a larger amount of the hematin pigment is scattered throughout the parasite. One can recognize both male and female types of this mother sexual cell, the difference being that in the male the nuclear material is more scattered, and therefore stains less definitely. These female and male gametocytes are the latest stages found in the human body. Subsequent stages develop only after the gametocytes get into the stomach of the acceptable female anopheline mosquito.

The most important diagnostic feature to recognize in the tertian parasite is the enlarged parasitized cell, which becomes very definitely pale as its substance is taken up by the parasite.

The Quartan Parasite (Plasmodium malariae).—The parasitized red blood cell does not increase in size. Occasionally it is even somewhat shrunken. It does not become paler in color. Frequently it is a bluish slaty hue, but this condition does not develop until the parasite has increased somewhat in size. The quartan parasite never gets as large as the cell which it parasitizes. There is more of a tendency also for the vacuole to be small compared with the amount

of solid cytoplasm. When the process of schizogony begins, the number of units which develop is few, seldom more than eight, compared with the larger number in both of the other two species. These nuclei usually have a much more symmetrical arrangement than they do in tertian infection, so that they appear like the petals of a rose around the center. Meanwhile, as in the other two species, a considerable amount of hematin pigment is deposited within the quartan plasmodium.

The so-called banded form, another variety of the quartan trophozoite, is relatively common, although not as common as it is figured in the textbooks. It may develop directly from the merozoite which has just entered the red blood cell; it continues to grow by a widening of the band so as finally to occupy a total of from two-fifths to one-half of the area of the red blood cell. The gametocyte stages of the quartan plasmodium are ovoid bodies, more nearly filling the parasitized red blood cell than the trophozoite stages. They have a large amount of pigment scattered throughout their cytoplasm. The female gametocyte has a definite nucleus, while the male gametocyte may have nuclear material so scarce and so scattered that it is difficult to recognize it in the ordinary stained film.

The Estivo-autumnal Parasite (Plasmodium falciparum).—There may be from one to several of these trophozoites inside each parasitized red blood corpuscle. Sometimes the ring may be peripheral in position, looking like a minute blister on the side of the cell, with the chromatin dot on the elevation of the blister. This early stage of estivo-autumnal infection is the only asexual form found in ordinary peripheral blood. The later trophozoite stages and the schizont are found almost without exception in the blood of the deeper viscera, including the maternal side of the placenta. Occasionally, however, these later stages in schizogony are found in peripheral blood, in which event there is a poor prognosis for the case. The late trophozoite is never as large as even the moderately developed trophozoite of tertian or quartan infection. It is usually oval in shape, quite regular in outline, and has a mass of cytoplasm opposite the nuclear or chromatin element. When it divides, one can recognize from eight to twelve up to a maximum of thirty-two nuclear elements, these being arranged around the center of hematin pigment. In the estivo-autumnal parasite, there are found from time to time stiplings in the nonparasitized part of the red blood cell. These are similar in type to those found in the tertian infection, but are called Maurer's dots.

The early gametocyte stages are also not found in peripheral blood, but in the deeper visceral circulation. These early gametocytes of estivo-autumnal malaria are broadly oval in shape, and at this stage male and female forms cannot be differentiated, although when they get out into the peripheral blood the two sex types can be distinguished. Both of them when ripe are of the socalled crescent shape with a thin membrane around the parasite which really is the pellicle of the parasitized red blood cell. Within the crescent one can recognize hematin pigment and concentrated nuclear material in the female gametocyte and more diffuse nuclear material in the male gametocyte. At a somewhat later stage, the pellicle of the parasitized red blood cell breaks down and the crescent or bean shaped gametocyte is set free into the blood stream.

THICK BLOOD FILMS

The thick blood film is a method of diagnosing malaria which has been accepted by many laboratorians as much more valuable than the thin blood film. This film is prepared by taking two or three drops of blood from the ear or finger, or in the case of the small child, from the great toe, placing it in the center of a clean slide, and distributing it with the corner of another clean slide, so that it will occupy the area of a quarter, and also so that the center will not be thicker than the margin. This is then allowed to dry thoroughly. In moist tropical climates, it is frequently necessary to place it in a drying oven for an hour or so. It is then dehemoglobinized and stained at the same time by using Giemsa's stain. It is, however, quite possible to use dilute Wright's stain in exactly the same way, by taking the stock Wright's solution, diluting it one part to thirty with neutral distilled water. The film is placed in a staining jar and left there for a half hour to an hour. When the film is taken out, it is thoroughly washed in water and dried. An examination will indicate that the red blood cells have been dehemoglobinized so that they appear as delicate ghosts on the film, although all of the leucocytes, as well as any malarial parasites which were present in the red blood cells, will be properly stained. The advantage of this method is that one can see right down through several superimposed layers of red blood cells. In other words, you have a concentrate of malarial parasites, which, if they had been stained by the ordinary process, would be so dense that the parasites could not be discovered. The parasites in this type of preparation are not flattened as on the thin film, so that it is necessary to acquire some experience with the method before accurate diagnosis can be made.

The thick film is particularly valuable in cases that have been found constantly negative by the thin film method; the writer has been able to vouch for this fact in several cases where the clinical symptoms were those of a malarial infection, but where the thin blood film was consistently negative.

3. *Sarcosporidia (Class undetermined, possibly Sporozoa).*

A few cases of infection with a member of this group, Sarcocystis hominis, have been found in human muscle tissue. It seems likely that the infection in man is really a casual inoculation from domestic or wild animals which more commonly harbor these species of parasites. The organisms have been recovered from heart muscle, muscle fibers of the larynx, biceps muscle and tongue. They are bean or sickle shaped and occur in large tubular clusters between the muscle bundles. The individual spores may measure up to 16 μ by 9 μ and the bundles as much as 25 mm. in length. The human cases reported have mostly been determined from necropsy material, although Darling (1919) diagnosed one case from a biopsy specimen.

REFERENCES

General References

Craig, C. F.: A Manual of the Parasitic Protozoa of Man. Philadelphia, 1926, J. B. Lippincott Company, 569 pp.

Dobell, C., and O'Connor, F. W.: The Intestinal Protozoa of Man. London, 1921, Wm. Wood & Company, 211 pp.

Knowles, R.: An Introduction to Medical Protozoology. Calcutta, 1928, Thacker, Spink & Company, 887 pp.

Lynch, K. M.: Protozoan Parasitism of the Alimentary Tract. New York, 1930, Macmillan Company, 258 pp.

Wenyon, C. M.: Protozoology, 2 volumes, London, 1926, Bailliére Tyndall & Cox, 1563 pp.

Amebae

Boeck, W. C., and Drbohlav, J.: The Cultivation of Endamoeba Histolytica, Am. J. Hyg. **5**: 371-407, 1925.

Craig, C. F.: The Nuclear Structure of Dientamoeba Fragilis, J. Parasitol. **13**: 137-140, 1926.

Craig, C. F.: Observations Upon the Hemolytic, Cytolytic and Complement-Binding Properties of Extracts of Endamoeba Histolytica, Am. J. Trop. Med. **7**: 225-240, 1927.

Faust, E. C.: A New Type of Amoeba Parasitic in Man, Observed in North China, J. Parasitol. **9**: 221-226, 1923.

Jepps, M. W., and Dobell, C.: Dientamoeba Fragilis n.g. n.sp., A New Intestinal Amoeba From Man, J. Parasitol. **10**: 352-367, 1918.

Kofoid, C. A.: Councilmania Tenuis and C. Dissimilis, Intestinal Amebas of Man, Arch. Int. Med. **41**: 558-585, 1928.

Kofoid, C. A., and Swezy, O.: On the Free, Encysted and Budding Stages of Councilmania Lafleuri, a Parasitic Amoeba of the Human Intestine, Univ. Calif. Pub. Zool. **20**: 170-189, 1921.

Kofoid, C. A., and Swezy, O.: The Cytology of Endamoeba Gingivalis (Gros) Brumpt Compared With That of E. Dysenteriae, Ibid. **26**: 165-198, 1924.

Kofoid, C. A., and Swezy, O.: Karyamoeba Falcata, a New Amoeba From the Human Intestinal Tract, Ibid. **26**: 221-242, 1924.

Taliaferro, W. H., and Becker, E. R.: The Human Intestinal Amoeba, Iodamoeba Williamsi and Its Cysts (Iodine Cysts), Am. J. Hyg. **2**: 188-207, 1922.

Intestinal Flagellates

Hogue, M. J.: Waskia Intestinalis; Its Cultivation and Cyst Formation, J. A. M. A. **77**: 112-113, 1921. Studies on Trichomonas Buccalis, Am. J. Trop. Med. **6**: 75-88, 1926.

Kofoid, C. A.: On the Morphology and Behavior of Pentatrichomonas Arden delteili (Derrieu and Raynaud), Univ. Calif. Pub. Zool. **20**: 373-390, 1923.

Kofoid, C. A., and Swezy, O.: On the Morphology and Mitosis of Chilomastix Mesnili (Wenyon), a Common Flagellate of the Human Intestine, Ibid. **20**: 117-144, 1920.

Simon, C. E.: Giardia Enterica: A Parasitic Intestinal Flagellate of Man, Am. J. Hyg. **10**: 440-491, 1921.

Hemoflagellates

Chagas, C., and Villela, E.: Cardiac Form of American Trypanosomiasis, Mem. Inst. Oswaldo Cruz **14**: 3-54, 1922.

Napier, L. E.: A New Serum Test for Kala Azar, Ind. J. Med. Research **9**: 830-846, 1922.

Nicolle, C.: Culture des corps de Leishman isoles de la rate dans trois cas d'anemie splenique infantile, Bull. Soc. Path. exot. **1**: 121-126, 1908.

Sia, R. H. P.: Ray's "Hemolytic" Test in Kala Azar, China M. J. **35**: 397-399, 1921.

Thompson, J. G., and Sinton, J. A.: The Morphology of Trypanosoma Gambiense and Trypanosoma Rhodesiense in Cultures, Ann. Trop. Med. Parasitol. **6**: 331-356, 1912.

Young, C. W., and Van Sant, H.: Leishmania Donovani in the Peripheral Blood, J. Exper. Med. **38**: 233-256, 1923.

Malaria plasmodia

Craig, C. F.: The Classification and Differential Diagnosis of the Aestivo-Autumnal Malaria Plasmodia, Am. J. Trop. Med. **1**: 57-96, 1921.

Thompson, J. D., and Woodcock, H. M.: The Parasites of Malaria, Byam and Archibald's *Practice of Medicine in the Tropics*, **2**: 1516-1546, 1922.

Sarcosporidia

Darling, S. T.: Sarcosporidia in Panama, J. Parasitol. **1**: 113-120, 1915. Sarcosporidiosis in an East Indian, Ibid. **6**: 98-101, 1919.

Ciliata

McDonald, J. D.: On Balantidium Coli (Malmsten) and Balantidium Suis (sp. nov.), Univ. Calif. Pub. Zool. **20**: 243-300, 1922.

INTESTINAL PROTOZOA IN CLINICAL MEDICINE*

By Kenneth M. Lynch, M.D., LL.D., Charleston, S. C.

THE position held by intestinal protozoa in clinical medicine is a confused one, commonly varying from one extreme to another among practitioners. The question of the relation of these protozoa to health and disease is of great and world-wide importance.

It is indicated by reports from various and widely separated sources that the general incidence of intestinal protozoa may be conservatively estimated at from 20 to 25 per cent or higher. It appears that the conditions for their transmission are more favorable in the warm countries but reliable studies made in north temperate regions and in institutions with modern sanitary facilities have shown that hot climate and unsanitary living habits are not essential to their spread.

In some sections of this country little or no attention is paid to intestinal protozoa in the diagnosis or treatment of disease. In other places the mere presence of one or more of these organisms is sufficient grounds for a specific diagnosis and therapeutic attack. With some medical men diarrhea or dysentery is the only condition associated with intestinal protozoan parasitism, while others have included among their effects such diseases as iritis, arthritis, Hodgkin's disease, cholecystitis.

It appears that much of the confusion concerning their status results from too much generalization in our consideration of them; to think or speak of them as a class, as protozoa, or as amebas or flagellates or ciliates, is of the same order as would be a similar consideration of bacteria as a class. The necessity of properly identifying and of studying the effects of the individual species concerned should be clearly forced upon us by the definite knowledge that one species of ameba is a disease producer while another is not.

For improvement in the confused status of the intestinal protozoa of man it is necessary to improve the knowledge and familiarity of the medical profession in the subject. Particularly is it important to have improvement in laboratory identification of these organisms. It is a widespread condition that laboratorians, medical and nonmedical, who are not well qualified for this work are given the responsibility of it.

Flagellates may be satisfactorily identified in a fresh liquid stool. The amebas are best identified in the encysted stage, which occurs mainly in the naturally formed stool. Many errors are made in naming an ameba found in liquid stools, of dysentery, diarrhea, or produced by a purgative, where it is present usually in the active form. For routine stool examination, unless there is diarrhea, it is best to study first a fresh stool produced by a saline cathartic. If an ameba is found and the cysts are not seen, one who is not an expert protozoologist should wait for its identification in a formed stool when the cyst

*From the Department of Pathology, Medical College of the State of South Carolina

will usually appear. However, in the case of clinical amebic dysentery, with the typical blood tinged mucous stool containing the active amebas with ingested red blood corpuscles, it is not advisable to wait for more accurate identification of the ameba before instituting proper treatment.

THE AMEBAS

Among the amebas occurs the protozoan of outstanding importance in clinical medicine, Endamoeba histolytica.

Once considered of importance only in the tropics, this parasite is of world-wide distribution. In temperate or colder regions its incidence is usually less but it has been found wherever search has been made for it. The reported incidence in different surveys varies widely, from around 1 per cent to 20 per cent and more, the variation in reliable studies apparently depending largely on the nature of the examined people, as to the sanitation of their environment and whether they are well or the subjects of intestinal disease. The greater incidence is naturally where the protection of man from excreta of his own kind is poorest, this usually being in tropical or subtropical regions. In institutional life, with its close personal contact, it is likely to be comparatively high. In rural districts without modern sanitary facilities it is also apt to be high. In modern communities with proper sewage disposal and protection of food and drink it is low. At a rough estimate its general incidence in a country like the United States of America is around 10 per cent. In the modern city it is around 3 per cent, according to my experience. Close personal contact with infected persons and the food handler carrier probably furnish the main means of spread.

The cyst of the organism is the infective stage and prevention of spread of the infection involves prevention of this body from reaching the mouth of man.

When this ameba reaches the intestine of man in viable state, it may produce amebic dysentery or chronic intestinal amebiasis, and there is some indication that it may result in a symptomless carrier state.

The known effects of the organism depend on entry into the intestinal wall with variable penetration, ulceration, and the possibility of transportation through the circulation to near or distant tissues, particularly the liver. A humoral absorption of toxic products of the organism is indicated.

The involvement of the intestine is practically limited to the colon, extension to the lower end of the ileum sometimes occurring. When the penetration and resulting ulceration of the colon are active and extensive, they result in the clinical state of amebic dysentery. This affords the common conception of the results of amebic infection. Amebic dysentery is rather the comparatively uncommon acute phase or end-result of intestinal amebiasis.

What determines the occurrence of amebic dysentery in one infected person and of a symptom producing chronic colitis or of a symptomless state of parasitism in others, or why one of the latter may have an acute flare-up of the infection into dysentery, is unknown. The state of parasitism is constituted of a balance between invasive properties of parasite and resistance of host. When that balance is in favor of the parasite, we have active ulceration and dysentery. When in favor of the host, the disease is halted or held in check or perhaps invasion of the parasite prevented.

The diagnosis of amebic dysentery is not difficult. Its characteristics are frequent bloody mucous stools, with abdominal griping and tenesmus. In the stool, on proper examination, are found active amebas, usually containing red blood corpuscles, associated with mucus, blood, fecal débris, and pus in the advanced stages. These are sufficient for a diagnosis upon which treatment may be instituted.

The diagnosis of chronic intestinal amebiasis is not easy. The patient usually has a history of long-continued ill health with confusing symptoms of many kinds but particularly relating to the intestinal tract, "indigestion" as the term goes. Although attacks of diarrhea or even dysentery, alternating with periods of constipation, may be discovered in the history, they may not be more conspicuous than in other people of similar ailments. Vague abdominal pains, heaviness, minor tenderness over the colon, especially the cecum, flatulence, gaseous eructation, are common complaints. Generally there is underweight, a "tired feeling," a lack of energy, with possibly headaches and joint aches.

Even a working diagnosis can only be made by positive identification of Endamoeba histolytica in the stool, possibly only by repeated examination. In practice this amounts to finding and identifying the cyst.*

Since similar chronic complaints are very common and since a large percentage of the people concerned harbor amebas of some species, identification of Endameba histolytica is the essential for a diagnosis of chronic intestinal amebiasis.

During the course of amebic dysentery or chronic intestinal amebiasis amebic abscess may occur elsewhere, most commonly in the liver.

For a general summary of the treatment of all phases of intestinal amebiasis it may be stated that the measures used should be both specific and nonspecific, that the pathology of the disease should be borne in mind, and that "cure" is not obtained until the ameba is proved to be eliminated, by an extended series of stool examinations.

It is not possible here to go into the details of specific and nonspecific treatment. Emetine in some form, commonly with bismuth, is the "specific" of widest satisfaction, particularly in the acute phases. It is now supplemented by yatren and stovarsol, the former apparently particularly applicable in the acute phase and the latter in the chronic. In the acute phases and during active emetine treatment the patient should be confined to bed and the diet restricted. During treatment of the chronic phase it is not necessary to put the patient to bed, but the diet should be restricted to a bland well-balanced one. A reduction of carbohydrates, sufficient protein, and particular attention to green vegetables and fresh fruits are recommended. There is perhaps more influence of dietary factors in intestinal amebiasis, invasion, resistance, and treatment, than we now

*Study of amebic cysts for diagnostic purposes in medical practise amounts to looking for that of Endamoeba histolytica to the exclusion of the others. Amoeba cysts are rounded glassy or hyaline bodies, easily overlooked, sometimes very numerous, sometimes not. Search for them is facilitated by mixing the particle of stool on a slide with an iodine solution, such as Gram's iodine (iodine 1 part, potassium iodide 2 parts, water 100 parts). This stains them various shades of brown and makes them more conspicuous. It also allows the nuclei to become more visible, as colorless rings against a yellow-brown background. When a suspicious body is located with the low power lens, the higher dry magnification will usually reveal its structure. If the cysts show four round nuclei with tiny central karyosome it is Endamoeba histolytica. The main confusion is with the cyst of Endamoeba coli: it has eight nuclei. For even better and more prolonged study fresh smear preparations fixed in warm Schaudinn's solution while still moist and stained by one of the haematoxylin methods is to be recommended. Such preparations exhibit the nuclei much better.

realize. In anticipating recovery from this disease we must keep in mind that the organism is to be eradicated, ulcers must be allowed to heal, and constitutional effects be overcome.

OTHER INTESTINAL AMEBAS

The main concern about the other intestinal amebas is that they must be differentiated from Endamoeba histolytica. They are: Endamoeba coli, the most common ameba of the intestine, and Endolimax nana, Iodamoeba butschlii, and Dientamoeba fragilis, these three being relatively uncommon and not so subject to the frequent confusion with Endamoeba histolytica as is the first. None of these is now under serious suspicion of producing disease.

FLAGELLATES

There are three well-established species of flagellates commonly found in the intestine of man, Trichomonas hominis, Chilomastix mesnili, and Giardia intestinalis. There are others less commonly seen, Embadomonas intestinalis, Tricercomonas intestinalis, and possibly Enteromonas hominis.

Probably the majority of protozoologists most familiar with these organisms now believe the class to be harmless to their hosts, although some hold the question open. Among medical persons there exists the greatest confusion as to the different genera and species and as to their effects on their hosts, a great many practitioners believing that at least some of them are disease producers. To these such terms as "flagellosis," flagellate infection, flagellate diarrhea, and flagellate dysentery relate to clinical states of more or less definiteness in their minds.

There exists no reason why any interested examiner may not easily identify the common flagellates as they occur in the stool and this should be the first objective of those who would study this class of parasitism.

The problem of the effects of these flagellates upon man has reached a stage of *impasse*, when evidence given on either side becomes merely an argument. None of the class has been shown to be an actual tissue invader or destroyer, none has been shown to produce any substance or bring about any state deleterious to the host. There is no positive direct evidence on the question; it is all clinical circumstantial evidence based on the occurrence of indefinite symptoms of abnormal states associated with the presence of the parasites. Such evidence, if it were definite or characteristic, would weigh more than it does. There is no typical or characteristic clinical or pathologic complex to be related to the presence of any of them.

Trichomonas hominis is an inhabitant of the large intestine. It is a common parasite found in fresh stool examination. It occurs from childhood to old age, but is apparently not a parasite of infancy. Continued study of the parasite should be carried on by those in position to do so. Practicing physicians should be careful that they are not led away from making another diagnosis by the finding of this organism in the stool.

Chilomastix mesnili is commonly confused with Trichomonas. It is also a common inhabitant of the large intestine. It becomes more common as the age of the individual advances and may live for years in the bowel. It is commonly

found during a state of lowered gastric acidity, or constipation, or the syndrome of chronic cholecystitis, as clinically diagnosed. To consider the clinical aspects of the disease, states commonly associated with its presence may be more to the point than too much attention to the parasite.

Giardia intestinalis is a common inhabitant of the upper small intestine. It is more common in childhood than in later life and decreases in frequency as age increases. In children it is commonly associated with a state of diarrhea, and the younger the child apparently the more commonly this occurs. It is consequently considered to be the cause of diarrhea in children, although studies which indicate this have not been sufficiently well controlled to make this certain. As the age of the host increases the individual seems able to control the parasite and apparently eliminates it commonly.

Its connection with the clinical state of enteritis and diarrhea apparently is lost in older people and the parasite then commonly appears to be harmless. Whether this is by immunization of the host or whether related to the difference in food and in digestive action between early and later life furnish interesting speculation about the unknown.

The diagnosis of this infection depends upon finding the parasite in the stool, although it is occasionally seen in duodenal drainage. In formed stools only the cysts appear. These are small and may be overlooked by the inexperienced examiner. The active form is usually seen in the stool of diarrhea.

THE CILIATES

Balantidium coli is practically the only ciliate of the human intestine, and it is a rare parasite in this country. Its natural host is probably the pig, and human infection probably comes from transmission from this animal. Few authentic cases of balantidial infection have been reported in this country. It has been reported as relatively common in the Philippine Islands and in Central America.

It is easily identified, but is very apt to be confused by the inexperienced with free-living ciliates which may occur in human stools after their passage.

This parasite is a potential and actual disease producer in man, balantidiosis of the intestine of man having a significance akin to intestinal amebiasis.

The acute phase of the disease is the unusual consequence of infection, the acute attack, or the end-result. In this condition the organism is a tissue invader and may give rise to deep ulceration of the colon associated with frequent bloody mucous stools and abdominal griping, but sometimes there is merely a watery diarrhea. Again, there may be ulceration without dysentery or diarrhea.

Much more common than the state of balantidial dysentery is that in which there is a chronic infection without dysentery or even without any apparent disturbance due to it. It is unknown whether in the "carrier" state without symptoms of disease the organism is purely a lumen dweller. The state of infection in which there is a chronic or mild and continued process of invasion and ulceration is less known than the comparable condition of chronic intestinal amebiasis, although when symptoms of progressive disease are present, they may be more pronounced. Not uncommonly there is a chronic and intractable diarrhea, and anemia and emaciation may be conspicuous.

In the treatment of balantidiosis stovarsol has been reported as efficacious in destruction of the organisms in the intestine. Benzyl benzoate has also been reported to be useful. Bismuth subnitrate is used to relieve cramps and diarrhea. Colonic irrigations of concentrated solutions of quinine have been reported to relieve the symptoms of balantidial dysentery and to reduce the number of parasites, even when not effecting a radical cure. The reduction of carbohydrates, the use of a milk and egg diet during the treatment of dysentery, and the use of fresh fruits and vegetables are dietary measures of importance.

As in the case of intestinal amebiasis the treatment of this condition consists of measures aimed to destroy the parasites, to aid in healing of ulceration, and to overcome constitutional effects. It should be borne in mind that a cure is not obtained unless the organisms are eliminated.

COCCIDIA

Although coccidia are protozoa which may be parasitic in the intestine of man, the infection is so rare in most countries, including the United States, that little attention will be given it here.

No definite clinical state is associated with coccidiosis in man, the host being usually in apparent good health, although the organism passes a part of its life cycle in the epithelium of the intestine, presumably the small bowel. It is indicated that there may be a temporary diarrhea and abdominal discomfort but that the infection is self-limited and that spontaneous recovery is complete. The species infecting man is Isospora belli, usually erroneously called Isospora hominis.

BACTERIAL MUTATIONS*

By F. d'Herelle, M.D., and Ruth Beecroft, M.A., New York, N. Y.

SEVERAL investigators within the past few years have studied the variations which strains of bacteria may present in the course of their cultivation on artificial media. Their results have been accepted by some and denied by others. If both conceptions are correct one may conclude, first, that bacterial variation is only an occasional phenomenon—some strains may vary, but the majority do not—and second, that since variation occurs only with certain strains, the cause of this phenomenon is foreign to the bacterium. When it is present variation occurs, when absent the bacterial culture remains stable.

Variations which occur among living beings may result, in general, from three different phenomena:

1. The existence of a life cycle such as one finds in the case of protozoa and insects. This phenomenon is easy to recognize for it is cyclic; the variations are "ordinate." All the characters vary together as a whole. Moreover, in a species which has a life cycle, all the individuals suffer the same variation when placed in identical environments.

2. The adaptation to new external conditions. This phenomenon is not cyclic. One can produce easily such variations with bacteria when they are cultivated in what one might term "abnormal" media; but generally such variations are transitory. The character "sporulation" is the only one, we think, that undergoes a permanent modification after a strain has been cultivated or exposed to the presence of certain antiseptics.

3. Adaptation to new internal conditions. New internal conditions always result from the subjection of an organism to a parasite. Such parasitism is termed "symbiosis" when the host adapts itself to this new condition. This adaptation is always correlative to modifications of the characters of the host (as well as modifications of the characters of the parasite). The mutations which develop due to symbiotic relationships have been studied very extensively by botanists during the last fifty years, especially since the paramount studies of Noel Bernard.[1] As far as variation is concerned, bacteriology is a division of botany, and it is remarkable how very few bacteriologists know anything about the researches dealing with variations done by botanists.

To which of these phenomena is the variability of bacteria related? In other words, which one is the cause of the variations?

It appeared to the senior author, from experiments carried on since 1920,[2] that the best manner to get at the cause of bacterial variations was to take a nonmutating strain and infect it with bacteriophage, in other words to produce an experimental "bacterium bacteriophage" symbiosis, and to observe what takes place when a culture subjected to such an infection is studied continually over a period of years.

*From the Division of Protobiology, Yale University School of Medicine.

Our studies have been concerned with the *Salmonella enteritidis* because of its pathogenicity for the mouse. Variations of the character "virulence" is certainly one of the most important. In fact, from the "man" point of view the most important. The original strain was strain 904 obtained from the American type collection culture, isolated in 1924 from the commercial rat "Danysz virus." At first we made a series of purifications by isolated colony study. Preliminary tests were performed in order to find out whether our strain was free from bacteriophage. Repeated experiments failed to show the presence of a phage. During the last two years we have used all the known methods to try and extract a phage from this strain but without success. More than 300 transfers of that strain have been made on various kinds of media, including those described by Hadley, but in no case have we observed the least variation; its characters have remained stable.

In December, 1929, we produced the lysis of a culture of that stable strain of Salmonella enteritidis by adding to the culture 0.01 ml. of a bacteriophage originally isolated from the stools of a convalescent typhoid patient, and since that time cultivated at the expense of *Eberthella typhi*. The lysed culture remained clear for eight days and then a slight secondary growth appeared. On the fifteenth day after the lysis appeared infusion plates and Endo agar plates were inoculated by spreading over their surfaces drops of the secondary culture. The colonies which were macroscopically different from those of the pure Salmonella were inoculated into infusion broth; from these cultures, new isolations were made in infusion agar and cultures derived from colonies which were completely resistant to the phage were kept for future experiments. In this manner we obtained 21 different strains. We could have selected more strains for every one of the 21 strains have continued to present new variants from time to time but had we done so our study would have become too complicated. In fact we could not study completely more than eight strains, the first eight isolated.

For lack of time we cannot describe in detail the aspect of these cultures. We shall merely state that the colonies of Mutant I are smooth, small (1 mm. after twenty-four hours) and refractive; colonies of Mutant III are large (3 to 4 mm.), opaque and as mucoid as those of the Friedlander bacillus. Mutant I cultured in infusion broth causes a slight homogeneous turbidity; Mutant II, on the other hand, in the same medium shows no turbidity, the broth remains clear, the culture forming clumps in the bottom of the tube. Mutant IV cultured in the above mentioned medium develops a marked turbidity. Every one of the eight mutants is different.

Immediately after isolation all of the mutants were entirely resistant to the action of the original bacteriophage. However, filtrates from these cultures contained a phage active against the original Salmonella. Hence, every mutant was a carrier of bacteriophage.

After 10 subcultures (in broth and on agar) Mutants II, IV, V, and VII continued to carry the phage, but at this time were sensitive to the original phage. Mutants I, III, VI, and VIII also carried the bacteriophage, but were resistant to the original phage.

After 150 subcultures the eight mutants were sensitive to the original phage. Also the presence of phage in broth culture filtrates of Strains I, III, V, and VI could be easily recognized for these filtrates produced lysis of the primitive Salmonella.

A series of experiments were carried out to determine whether Mutants II, IV, VII, and VIII were free from phage. Several isolated colonies from each of the mutants were carefully studied in a number of preliminary experiments. The results of the main series of experiments demonstrated: (1) that from one of six colonies isolated from Mutant II, we could show the presence of a phage active against the original Salmonella; (2) that from thirty colonies picked from Mutant IV only one could be shown to be a phage carrier; (3) that from 100 colonies picked from Mutants VII and VIII not one colony could be proved to carry bacteriophage active against the primitive strain.

It seems therefore, that certain colonies are carriers of bacteriophage while others are free; but further investigation proved that this was not the case. Actually in colonies which appear to be free from phage the phage is "latent." This was true not only in the colonies isolated from Mutants II and IV but also in I, III, V, and VI.

This may be demonstrated in the following manner: A drop of culture of Mutant III is spread over the surface of an infusion agar plate; from the resulting growth a Friedlander-like colony was isolated. This colony was cultured in broth and the resulting growth passed through a filter. This filtrate possessed no action against the original Salmonella. A drop of this broth culture (before filtering) was spread over the surface of an agar plate. After incubation the majority of colonies were Friedlander-like. Interspersed among the colonies were a few nonmucoid colonies and some tiny colonies. Two colonies of each of the three types were inoculated into broth and after a twenty-four-hour incubation period were filtered. The filtrate from one of the Friedlander-like colonies and from one of the nonmucoid type colonies produced lysis of the original Salmonella. The filtrates of the other four colonies were negative.

We conclude, therefore, that in these mutations all of the colonies are phage carriers, but that in some of them the phage is active, in others it is "latent." The number of colonies (that is to say the number of bacteria) which carry an active phage varies according to the mutant. In our series with Mutant VI approximately every one out of two individuals carries an active phage; with Mutant IV only one out of thirty. The rate of phage reproduction is different also. It is rapid in the case of Mutant VI; very slow in Mutant IV. Thus it appears that in the case of Mutant VI there is a rapid reproduction of the phage at the expense of numerous bacteria, while few absorb it, then many phage corpuscules are free in the liquid and pass in the filtrate, on the contrary, in Mutant IV, a few bacteria reproduce the phage and many absorb it, with a result that no corpuscules are free and found in the filtrate.

But there is another phenomenon which conceals bacteriophage.

We could never detect the presence of a phage acting upon the primitive Salmonella in Mutants VII and VIII. However, the action of filtrates of these mutants were tested against cultures of the other mutants, and it was found that Mutant VII contained a phage acting only upon Mutant II.

A broth culture of Mutant VIII was streaked on an agar plate and 20 colonies picked for study. Broth cultures of these colonies were filtered, and the filtrates tested upon cultures of the other mutants, as well as upon the original culture. Only one was active and this one lysed a culture of Mutant VII. It appeared that the bacteriophage had lost its pathogenicity for the primitive strain but retained its virulence for certain mutants.

All of these mutants continued to be carriers of phage after 150 subcultures, and still persisted in showing "dissociations." As an example of this let us consider for a moment Mutant III. This particular organism produced typical Friedlander-like colonies on agar plates, and even after 50 subcultures there was no change noticed in these colonies. After 150 subcultures we did observe again "dissociations," 90 per cent of the colonies were opaque, Friedlander-like; 2 per cent were transparent, Friedlander-like; 3 per cent were large, non-Friedlander; and 5 per cent were tiny colonies.

In fact "dissociations" are continual in strains which are bacteria bacteriophage symbiosis in contrast with the original bacteriophage-free Salmonella which does not vary. The phenomenon is not a simple one, and under natural conditions, it must be still more complicated, since in nature, variations take place not because of the action of one strain of phage (as in our experiments), but because of the action of several strains, for in nature bacteriophages are as ubiquitous as bacteria. We have some proof of this, for all of these mutants began to show new dissociations when subjected to the action of a new phage (one isolated from sewage and possessing a powerful action).

AGGLUTINABILITY

An antiserum having a final titer of 1:3200 was prepared by injecting 3 rabbits with the original strain of the Salmonella.

Table I gives the results of agglutination tests after 10 subcultures.

TABLE I

AGGLUTINATION OF THE MUTANTS AFTER 10 TRANSFERS

	1/100	1/400	1/800	1/1600	1/3200
Typhi-murium	++++	++++	++++	++++	++++
Mutant I	++++	++++	++++	+++	++
II	++	++	+	–	–
III	–	–	–	–	–
IV	–	–	–	–	–
V	+++	++	++	++	+
VI	++++	++++	++++	++++	++++
VII	–	–	–	–	–
VIII	++++	++++	++++	++++	++++
IX	++++	+++	+++	–	–
X	++++	++++	++++	++++	++++
XII	–	–	–	–	–
XIII	++	+	+	+	–
XIV	+++	++	+	+	–
XV	++	++	+	+	+
XVI	–	–	–	–	–
XVII	++++	++++	++++	+++	++
XVIII	++++	++++	++++	+++	++
XIX	++++	++++	++++	++++	+++
XX	++++	+++	++	++	++
XXI	++++	+++	+++	–	–

After 100 subcultures: Mutants I, VI, and VIII completely agglutinated at a titer of 1:3200. Mutants IV, III, V, and VII were not agglutinated in a dilution of 1:100. For Mutant II, agglutination was only partial from 1:100 up to 1:800.

Adsorption tests showed that the nonagglutinating mutants do not even adsorb the agglutinins (Table II).

TABLE II

ADSORPTION OF AGGLUTININS: SERUM ANTITYPHI-MURIUM; 2 ADSORPTIONS BY MUTANT

		AGGLUTINATION OF TYPHI-MURIUM: 1/100	1/1000	1/10000
Mutant	III	++++	++++	+
	IV	++++	++++	++
	VII	++++	+++	+
	XII	++++	++++	++
	XVI	++++	+++	++
Control:	unadsorpted on Typhi-murium	++++	++++	++

FERMENTATION STUDIES

After 10 and 20 subcultures, fermentations are as shown in Table III.

TABLE III

		DEXTROSE	LACTOSE	MANNITE	SUCROSE	DEXTRINE	SALICINE	MALTOSE
Orig.	Salmonella	AG	—	AG	—	AG	—	AG
Mutant	I	A	—	A	—	—	—	A
	II	AG	—	AG	—	A	—	AG
	III	AG	—	A	—	—	—	AG
	IV	A	—	A	—	A	—	AG
	V	AG	—	AG	—	AG	—	AG
	VI	A	—	AG	—	AG	—	AG
	VII	A	—	AG	—	AG	—	AG
	VIII	A	—	AG	—	A	—	AG

After 60 and 150 subcultures, fermentative characters of II, VII, and VIII are normal, same as original Salmonella. Mutant III is normal except that it does not produce gas in mannite medium. Fermentative characters of Mutant I have remained abnormal, as they were after 10 subcultures.

VIRULENCE

The virulence of the original strain has been tested repeatedly on mice and has not varied during the course of these experiments. One hundredth milliliter of a twenty-four-hour broth culture administered by mouth to ten mice killed all of them within seven to thirteen days. A dose of 0.001 ml. kills 6 to 8 out of ten mice within the same period. The virulence of the mutants has been tested after 10, 100, and 150 transfers.

In the following experiments we have considered our mutants to be non-virulent when ten mice each receiving a 0.1 ml. of a twenty-four-hour culture by

mouth still survive after a period of thirty days. These mice were then given a 0.01 ml. of the original Salmonella in order to determine whether their survival was due to a natural resistance. No such natural resistance was ever demonstrated.

After 10 subcultures: Mutants I and III are avirulent (all 10 mice survive). Mutants VI and VIII (all 10 mice die) are as virulent as the original strain. Further experiments with Mutant VIII done with doses of 0.001 and 0.0001 ml. show that its virulence is about the double that of the virulence of the original Salmonella. Mutants II (4 mice die), IV (2 mice die), V (3 mice die) and VII (5 mice die) are all less virulent than the original culture.

After 100 subcultures: Mutants I and III are still avirulent and IV and VII are also avirulent. Mutants II (4 mice die) and V (1 mouse dies) are slightly virulent. Mutants VI and VIII tested with doses of 0.01, 0.001, and 0.0001 ml. prove that in the case of Mutant VI the virulence has decreased slightly, while the virulence of VIII is still greater than that of the primitive strain.

After 150 subcultures: Mutants I, III, and IV are avirulent. II only slightly virulent (1 mouse dies). V and VII weakly virulent (2 and 3 mice die). VIII virulent, but not hypervirulent (8 mice out of 10 die) after infection with 0.01 ml. VI virulence has increased (10 mice out of 10 die) after infection with a dose of 0.001 ml.

The question of what happens to avirulent mutants after they have been ingested by mice next occupied our attention and a series of experiments were done in an attempt to answer this question. A series of ten mice were infected with 0.1 ml. of a culture of Mutant I. Thirty days later these mice were sacrificed and their splenic pulp spread over the surface of agar plates. All were bacteria free. When the same procedure was repeated using as a test culture Mutant III, the result was different. Four of the ten spleens examined yielded positive evidence of contamination with Mutant III.

A series of 20 mice received by mouth 0.1 ml. of a culture of Mutant III. Forty-two days later one of the mice died and an organism similar to Mutant III was recovered from the heart's blood and spleen of this animal. This organism showed no increase in virulence. Seven of the remaining nineteen died during the summer but no examination was made. On November 10 of this year (seven months after the original infection), a mouse died and from the heart's blood a pure culture of an organism similar to Mutant III was isolated. The remaining eleven were killed seven months after the infective dose was given; cultures of a bacterium similar to Mutant III were obtained from the spleen of four of these mice.

From these experiments we conclude that Mutant I is absolutely avirulent, whereas Mutant III shows some capacity to infect mice. However, in contrast to the original Salmonella, which produces an acute disease, Mutant III produces a chronic one.

Mutations brought about by bacterium bacteriophage symbiosis are not only a product of laboratory experimentation, but occur also in nature due to the same cause. Two examples may serve to support this fact. During the course of

this investigation in three batches of mice which we received, one or two mice were sick. Several days later all the remaining animals appeared to be in good condition. When several were sacrificed, however, we isolated from the spleens of these mice mutants contaminated with phage. Their characteristics, including virulence, were similar to those of our Mutants I, V, and VII. Even more striking is the experiment with another strain of Salmonella. As is well known, the Pasteur Institute of Paris prepares a virus which is distributed for the destruction of rats and mice. This virus is a culture of Salmonella enteritidis (Danysz virus) which came originally from the same source as our primitive Salmonella. The latter, however, has been cultivated on artificial media, while the Paris strain has undergone many repeated passages through rats and mice in order to exalt its virulence. The strain is now hypervirulent and will cause an acute fatal disease even in wild rats when they ingest this organism. However, the Pasteur Institute strain is not a pure culture, as we have recognized, it is a mutant and contains a phage inactive upon our primitive Salmonella but strongly active upon our Mutant VII. Apparently the exaltation of the virulence of this Pasteur Institute strain is not the product of animal passage, but the result of a sudden mutation which occurred by chance in the intestinal tract of one of the animals used in the serial passages. The exaltation of virulence by this means is probably very rare in nature, a decrease in virulence very common.

We have also experimental evidence to show that carriers of *certain* of our mutants are protected against ingestions of the primitive virulent Salmonella. Twelve mice were fed daily for eight days 0.1 ml. of a culture of Mutant I. They then received by mouth 0.01 ml. of the virulent primitive Salmonella. Following this, they again received by mouth 0.1 ml. daily for ten days a culture of Mutant I. Eleven and fourteen days after infection with the original virulent Salmonella two mice died. The other ten survived. All six control mice died within eight to thirteen days after infection.

Twelve mice were infected with 0.01 ml. of the virulent Salmonella and thereafter received 0.1 ml. of a culture of Mutant I daily for ten days. Three mice succumbed (nine, twelve and eighteen days after the infection). The remaining nine survived. All six controls died.

Similar experiments carried out with Mutant IV did not show any protective action.

In relation to the problem of carriers these experiments show that not all the carriers are infective. Some carry avirulent mutants, others mutants of low virulence, few of them carry mutants whose virulence is equal to that of the primitive strain, and finally some may carry mutants which may be hypervirulent. The latter are very rare in nature. We do not intend to generalize when we state the above, but it is certainly true in the case of mouse typhoid. Moreover, the senior author has demonstrated that in cholera, mutants are the principal cause of variations in the virulence of the vibrio, but in this case all the mutants are avirulent.[3]

This investigation shows that the principal cause of variations among bacteria is a result of a symbiosis and certainly not the result of a life cycle. The variations are not cyclic but appear in a very disorderly fashion. What is more

TABLE IV

CHARACTERS OF MUTANTS: 150 TRANSFERS

	AGGLUTINABILITY 1/100 - 1/800	VIRULENCE	FERMENTATIONS
Typhi-murium	++++	1	Normal
Mutant I	++++	0	Abnormal
II	+	1/100	Normal
III	–	?	Abnormal
IV	–	0	Normal
V	–	1/10	Normal
VI	++++	1	Normal
VII	–	1/10	Normal
VIII	++++	1/2	Normal

characteristic is that each character may vary by itself as an entity, without having any repercussive effect upon the other characters. If we consider for example the three characters "virulence," "agglutinability," and "fermentation," we observe that there is no relation between the variation of one of them and the variation of the others.

REFERENCES

1. Rayner, M. C.: Mycorhiza. London, 1927, Weldon and Wesley, Ltd.
2. d'Herelle, F.: Sur la resistance des Bacteries a l'action du Bactériophage, Compt. rend. Soc. de biol. **83**: 97, 1920.
3. d'Herelle, F.: Phenomenes coincidant avec l'acquisition de la resistance des bactéries à l'action du Bactériophage, Compt. rend. Soc. de biol. **84**: 382, 1921.
4. d'Herelle, F.: Studies on Asiatic Cholera, Indian Med. Research Memoirs, No. 14, February, 1930.

BACTERIOPHAGE IN CLINICAL MEDICINE*

By N. W. Larkum, Ph.D., Lansing, Mich.

WHETHER bacteriophage has a place in clinical medicine is a question that remains to be answered. That bacteriophage is in wide use in clinical medicine is a fact which as yet is not generally recognized. In Europe, especially in France, England, and Germany, one laboratory alone is distributing in the neighborhood of fifty liters a day. In South America, although no figures are available, the amount used is considerable. In the United States three biologic concerns are selling this product. In addition, a score or more private distributors are furnishing this material for experimental purposes. In Michigan the monthly output of bacteriophage has been about ten liters during the past two years. It is evident to all concerned that despite the entire absence of paid advertising, and in the face of strong opposition in some quarters, the number of patients receiving some form of bacteriophage treatment is relatively large and is increasing daily at a rapid rate.

The growing interest in bacteriophage as a therapeutic agent and the increasing demand from both patients and physicians presents a serious problem to those who are interested in the study of this principle. Because of conflicting experimental observations, enthusiastic and poorly controlled clinical application, and rapidly expanding commercial exploitation, a situation is developing which will, unless guided and checked, lead to the ultimate rejection of bacteriophage by all who make any pretense to the practice of scientific medicine. So infinite are the possibilities which the discovery of this principle has made apparent that it would be nothing short of a calamity to have its continued investigation retarded or even halted through the unbridled enthusiasm of those who, without regard to the mechanism of its action, make use of this principle whenever an opportunity presents itself.

Clinical observations without regard to theory have thus far constituted the chief impetus to the increasing use of bacteriophage. The glowing reports of successful treatment of conditions oftentimes regarded as hopeless, are characteristic of the sort of propaganda that has kept many doubtful biologic products in favor. That they have stimulated the use of bacteriophage is unquestionable, and it is equally certain that they should by no means be considered as valueless. Difficult as it is to draw conclusions from data of this type, the very weight of testimony in time becomes impressive.

Since clinical experience has been on the whole favorable and at the same time unconvincing, it appears that but little can be contributed by a detailed presentation of the material found in the literature. The methods have been similar in most instances, the results especially in the past few years almost invariably encouraging. On the other hand, the theory upon which therapy is based has been repeatedly attacked. The various properties of bacteriophage

*From Michigan Department of Health.

other than lysis have not received adequate attention, and animal experimentation has failed to provide support for clinical expectations. For these reasons it would appear that a presentation of some of the theoretical aspects of the problem would give a more accurate index of the position of bacteriophage in clinical medicine than would the more spectacular but less reliable figures of clinical experiments.

The socalled bacteriophage in general use for therapeutic purposes is in reality a rather complex mixture, one element of which is the bacteriophage proper. Each of the components of the mixture has certain biologic activities, some very well known, others as yet scarcely realized. The lytic principle itself possesses attributes other than lysis, among which may be mentioned ability to transform bacteria, i. e., to instigate bacterial dissociation, and ability to stimulate phagocytosis. The spectacular nature of the phenomenon of lysis has so far, however, overshadowed any other virtues possessed by the bacteriophage or by the other components of the filtrates in which the principle is contained, with the result that therapy has been based almost entirely upon the expectation of in vivo lysis. Almost without exception clinical experiment has been planned to provide the most favorable condition for lysis. This has resulted in a selection of cases to be treated and a choice of methods and procedures calculated to favor the lytic phenomenon.

One of the fundamental conditions imposed by the adoption of lysis as a basis for therapeutic application is that the infecting organism be susceptible to lysis in vitro. The effect of this concept upon procedure is obvious. The infecting organism must first be isolated. It must then be tested with known bacteriophage to determine whether lysis can occur. In the absence of lysis, an effort must be made to prepare a bacteriophage of maximum activity against this organism. In the event that a satisfactory bacteriophage is discovered, care must be used in the treatment to avoid the formation of antibacteriophagic antibodies. Since polyvirulent bacteriophages are not common and since the preparation of specific bacteriophages for given cases is difficult and frequently impossible, the number of patients suitable for treatment is limited. It is not uncommon to find antibacteriophagic serum in patients not previously treated with bacteriophage, which fact still further limits the possibilities.

Serious as are these objections to bacteriophage therapy, the failure to demonstrate that in vivo lysis can occur under the most favorable conditions has brought about what amounts to a complete collapse of the theory. Here, however, one must be cautious in generalizing, for conditions within tissues and in the circulating blood are not comparable with conditions in the intestine, the urinary bladder, and body cavities. Direct evidence of lysis in the intestine is difficult to obtain. In the isolated loop of intestine, Leitner[1] easily demonstrated bactericidal activity of bacteriophage. If this experiment has not been confirmed, it is equally true that no one has shown that lysis does not occur in the intestine. There is likewise much indirect evidence to the effect that here at least bacteriophagy can be expected. That lysis can and does occur in the urinary bladder has been demonstrated by Marcuse[2] and Larkum.[3] It is likewise not improbable that in the absence of extensive purulent exudate lysis can occur in other cavities.

In the circulating blood and within tissues, however, bacteriophagy is of doubtful occurrence. Applebaum and MacNeal[4] have recently demonstrated the inhibitory action of blood and pus. Krueger and Northrup[5] have indicated that a concentration of bacteriophage sufficient to cause lysis in blood or tissues is difficult to obtain and is probably never reached in ordinary clinical experience.

In the light of available information, one can only conclude that if the effectiveness of bacteriophage therapy is dependent upon in vivo lysis, its application is limited to enteric infections with some slight hope for kidney and bladder conditions. Even then the treatment is limited in application and must await considerable improvement before it can have universal utility.

Clinical experience whatever it lacks in scientific value has, however, clearly indicated the inherent possibilities in bacteriophage therapy in conditions where lysis could scarcely be expected to operate. The treatment of staphylococcus infections, especially of the skin, has been sufficiently encouraging to warrant continued and more exacting trial, and if lysis cannot be offered as an explanation for results, there are other and better explanations available.

In the preparation of bacteriophage, bacteria are lysed and as a result of lysis there must remain in the filtrate a certain amount of bacterial protein. The extent to which this protein is hydrolyzed to simpler compounds is not known at present and probably varies considerably according to the bacteriophage and bacterium used and the condition under which lysis occurs. Many investigators, Pache and Urech,[6] Schultz,[7] and others, contend that hydrolysis is marked and that very little bacterial protein remains. On the other hand the production of bacterial antibodies by these lysates has been repeatedly observed, notably by Arnold and Weiss,[8] Arloing, Josserand and Narbonne[9,33] and Larkum.[10] Furthermore, I have always found in lysates of staphylococcus that amino nitrogen represented only a small fraction of the total nitrogen present.

The clinical procedure recommended by the Michigan Department of Health and which has been used in more than 2,000 treatments is based upon the assumption that these bacteriophage filtrates are in reality vaccines, although we have not failed to provide a potent bacteriophage along with the proteins. We have always insisted upon inoculations in treatment regardless of the type of infection, and although no conclusions can be drawn as a result of our experience, we have the distinct impression that a very considerable immunity is established after inoculation. The production of antibacteriophagic sera is in our estimation a bogy not to be feared, and as time goes on it appears more and more possible that our doses (2 c.c.) are much too small. Further support for our beliefs is furnished by Stout,[11] who for a number of months has been using large and repeated inoculations with good results. From the same source we learn that bacteriophage prepared from freshly isolated strains of staphylococcus is much more effective than that prepared from old cultures. This checks with our own experience.

Local applications of bacteriophage in treatment of staphylococcus infections have been widely used and have been successful according to Rice,[12] who believes that this method is a combined bacteriophage and antivirus action. If one can attach any significance to the numerous reports of successful application of the principle of local immunity, it would appear that such results as

attend the local use of bacteriophage might be attributable to some such mechanism. In view of our present lack of information on the subject of antivirus, however, it is impossible to offer this as a contributing factor in the results.

Bacteriophage, it is claimed by d'Herelle,[13] Smith,[14] Arnold and Weiss,[15] and by Nelson,[16] has a remarkable opsonizing action. Bacteria which have had only a brief contact with the lytic principle are much more readily ingested by leucocytes than are normal organisms. With Knudsen, I[17] checked such results, and working with purified bacteriophage, we demonstrated that the bacteriophage itself rather than the proteins brought about the change. Opsonic indices as high as 40 have been reported, although the average for all experiments would be nearer 10. Whether this is significant from the standpoint of therapy has not been demonstrated.

The change in the bacterial substrate through the action of bacteriophage is as yet little appreciated in its therapeutic significance. Bacterial lysis has so far occupied the attention of investigators that the loss of virulence noted in secondary resistant cultures has received little attention. In fact, at first it was believed that those organisms which resisted the action of bacteriophage and developed following lysis had enhanced virulence. This view was held by d'Herelle,[13] who later revised his opinions, and the decreased virulence of such cultures has since been observed by numerous investigators. Hence it is entirely possible that even without lysis bacteriophage may considerably alter the course of an infection.

With so much controversy over the effectiveness of bacteriophage as a therapeutic agent and the mode of its action, it would seem that animal experiments might provide an answer, especially since controls could be easily assured. The one great objection to such experiments has been pointed out by d'Herelle[18] and is becoming more generally appreciated by immunologists. Purely artificial infections, although they have their place, cannot be accepted as a sound basis for immunologic or therapeutic conclusions. It is essential that experiments be carried on with infections to which the animals concerned are naturally susceptible. Even here artificially induced natural infections do not necessarily reproduce the conditions obtained during naturally transmitted disease. As a result, the situation with respect to controls is almost if not altogether as complicated as with human subjects.

Such experiments as have been conducted with animals have given rather variable results. The early experiments of d'Herelle[13] with fowl typhoid were not sufficiently extensive to be conclusive. His later work with barbone in buffaloes was apparently satisfactory, although Cowles and Hale[19] make the statement that in a personal communication d'Herelle claims the opposite. In this same paper Cowles and Hale summarize the situation with respect to animal experiments as follows:

"Pyle[20] working with an infection by Bact. pullorum, and Levy,[21] Topley, Wilson and Lewis,[22] Richet and Hauduroy,[23] and Bronfenbrenner and Korb,[24] studying mouse typhoid, all obtained unfavorable results as did Wollman[25] with Bacillus shigae and B. danysz. In experiments on plague in rats Doorenbos[26] found that bacteriophage . . . . exerted some protective action but Compton[27] failed to observe any such effect."

Cowles and Hale, themselves, were unable to protect white mice against anthrax by inoculations of bacteriophage, and Larimore and Harris[28] state that typhoid bacteriophage (in guinea pigs) exerted no prophylactic or therapeutic action on B. typhosus infection.

There are, however, a number of animal experiments which tend to demonstrate the relative efficacy of inoculations of bacteriophage as a method of choice in treatment and to attribute the results to the bacterial proteins involved. While Compton was unable to demonstrate any therapeutic value in plague bacteriophage, he was able to immunize rats against infection and concluded that failure to detect bacteriophage in immune animals leads to the conclusion that the immunity is antibacterial rather than protobiotic. Maslakowitz and Kasarnowsky[29] immunized rabbits with Shiga bacteriophage and found them protected against ten lethal doses of the bacteria. They, too, present evidence to show that the bacterial proteins rendered available by the bacteriophage provided the protection. Mistral[30] was able successfully to treat a paratyphoid infection in hogs by inoculation of bacteriophage and Arloing, Josserand and Narbonne[9] could protect guinea pigs against intraperitoneal inoculations of B. typhosus when these pigs received first serum from rabbits which had been immunized by inoculations of bacteriophage. This again favors the antibacterial rather than protobiotic action of bacteriophage therapy. Flu[31] was able to protect rats against 40 lethal doses of plague bacilli by giving preliminary inoculations of bacteriophage. Finally, Lucchini and Villa[32] state: "There is no evidence yet that bacteriophage itself has been of value therapeutically. The evidence favors the view that such therapeutic results as have been obtained were due to the action of the bacterial protein present."

Any careful reading of the literature must leave one more or less sceptical in his attitude toward bacteriophage therapy. On the other hand clinical experience and the wealth of possibilities inherent in the principle seem to demand the continuation of investigation. But investigation and not mere empirical application is required. While it is probably justifiable in instances where other measures are not usable and where bacteriophage has already given some indication of possible value that the patient should not be denied what may be of assistance, it is equally imperative that the methods of treatment be placed upon a sound scientific basis. It matters considerably whether the bacteriophage or the bacterial proteins are the effective fractions of the filtrates employed, for dosage, method of application, and preparation of the product depend upon an understanding of these facts. Study of the literature does not as yet offer a solution of the problem. It is highly essential that experiments be continued on both animals and human beings with a recognition of all the questions involved.

If several years of investigation into the problem of bacteriophage therapy has contributed anything at all, it has been this: That clinically gratifying results in spite of lack of knowledge of the principles involved have been the rule; that ingestion, local application or inoculation of bacteriophage oftentimes in large doses has so far as we can judge failed to complicate the infection or otherwise retard progress toward recovery in the patient; and that above all oppor-

tunity for carefully controlled clinical experiments which apparently can be carried out without detriment or discomfort to the patient is urgently needed.

REFERENCES

1. Leitner, Nikolaus: Versuche über Bakteriophagenerzeugung und Darmbakterzidie mittels einer isolierten Darmschlings in vivo, Ztschr. f. Immunitätsforsch. u. exper. Therap. **58**: 360-370, 1928.
2. Marcuse, K.: Grundlagen und Aufgaben der Lysintherapie, Deutsche med. Wchnschr. **50**: 334, 1924.
3. Larkum, N. W.: Bacteriophagy in Urinary Infections II Bacteriophagy in the Bladder, J. Bact. **12**: 225, 1926.
4. Applebaum, M., and MacNeal, Ward: The Influence of Pus and Blood on the Action of Bacteriophage, J. Infect. Dis. **49**: 225, 1931.
5. Krueger, A. P., and Northrup, John H.: The Kinetics of the Bacterium-Bacteriophage Reaction, J. General Physiol. **14**: 223-254, 1930.
6. Pache, H., and Urech, E.: Bacteriophage Antiserums, Schweiz med. Wchnschr. **56**: 275-278, 1926.
7. Schultz, E. W.: Personal communication, 1929.
8. Arnold, L., and Weiss, E.: Prophylactic and Therapeutic Possibilities of the Twort-d'Herelle's Bacteriophage, J. Lab. & Clin. Med. **12**: 20-30, 1926.
9. Arloing, F., Josserand, A., and Narbonne, A.: Pouvoir antigénique chez le Lapin des lysats de Bacilles d'Eberth obtenus avec un Bactériophage approprie, Comp. rend. Soc. de biol. **104**: 1246, 1930.
10. Larkum, N. W.: Bacteriophage as a Substitute for the Typhoid Vaccine, Abst. J. Bact. **17**: 42, 1929.
11. Stout, B. F.: Personal communication, 1931.
12. Rice, T. B.: Use of Bacteriophage Filtrates in Treatment of Suppurative Conditions, Am. J. M. Sc. **179**: 345-360, 1930.
13. d'Herelle, F.: The Bacteriophage and Its Behavior. Baltimore, 1926, Williams & Wilkins Co.
14. Smith, G. H.: Bacteriophage and Phagocytosis, J. Immunol. **15**: 125-140, 1928.
15. Arnold, L., and Weiss, E.: Antigenic Properties of Bacteriophage, J. Infect. Dis. **34**: 317-327, 1924.
16. Nelson, A. R.: The Effects of Bacteriophage Upon the Phenomenon of Leucocytosis and Phagocytosis, J. Immunol. **15**: 43-64, 1928.
17. Knudsen, J. R., and Larkum, N. W.: A Study of the Effect of Bacteriophage on Phagocytosis. Presented at Conference of Bacteriologists, Lafayette, Indiana, May 1, 1931.
18. d'Herelle, F.: Immunity in Natural Infectious Diseases. Baltimore, Williams & Wilkins Co.
19. Cowles, P. B., and Hale, W. M.: Effect of Bacteriophage on Experimental Anthrax in White Mice, J. Infect. Dis. **49**: 264-269, 1931.
20. Pyle, N. J.: Bacteriophage in Relation to Salmonella Pullora Infection in Domestic Fowl, J. Bact. **12**: 245-261, 1926.
21. Levy, M. M.: Bacteriophage in Bacillus typhi murium Infection, Comp. rend. Soc. de biol. **93**: 82-83, 1925.
22. Topley, W. W. C., Wilson, J., and Lewis, E. R.: Rôle of Twort-d'Herelle Phenomenon in Epidemics of Mouse Typhoid, J. Hyg. **24**: 17-36, 1925.
23. Richet, C., and Hauduroy, P.: Attempts at Immunization Against Mouse Typhoid With Bacteriophage, Comp. rend. Soc. de biol. **93**: 222-223, 1925.
24. Bronfenbrenner, J. J., and Korb, C.: On Variants of B. pestis caviae Resistant to Lysis by the Bacteriophage, Proc. Soc. Exper. Biol. & Med. **23**: 3-5, 1925.
25. Wollman, Eugene: Research on Bacteriophage, Ann. Inst. Pasteur **39**: 789-832, 1925.
26. Doorenbos, W.: Observations on Flu's Test in Immunization against Plague and my own experiences with Plague and Plague Bacteriophage, Nederl. tidjschr. v. geneesk. **2**: 5472-5482, 1929.
27. Compton, Arthur: Études sur l'immunité dans la peste expérimentale, Ann. Inst. Pasteur **45**: 754-767, 1930.
28. Larimore, O. M., and Harris, W. H.: Action of Bacteriophage in Experimental Typhoid Peritonitis, Proc. Soc. Exper. Biol. & Med. **26**: 754-756, 1929.
29. Maslakowitz, P., and Karanowsky, S.: Versuch zur Herstellung von Antigen en mittels Bakteriophagen Lysins, Mikrobiologichesky J. **3**: 151-236, 1926.
30. Mistral, Ch.: Le rôle du bacille paratyphique B et de son bactériophage au cours de la peste porcine, Comp. rend. Soc. de biol. **101**: 628, 1929.
31. Flu, P. C.: Immunizing of Rats Against Plague by Means of Extracts of Virulent Bacilli, Zentralbl. f. Bakteriol. **113**: 473, 1929.
32. Lucchini, C., and Villa, L.: Batteriofago e tossine bactericheche; Natura della terrapia col batteriofago, Boll. Inst. siero-terap. **5**: 231, 1926.
33. Arloing, F., Josserand, A., and Narbonne, A.: Emploi de Bactériophage pour l'obtention d'un serum antityphique, Comp. rend. Soc. de biol. **107**: 493, 1931.

OBSERVATIONS ON THE BACTERIOPHAGE III*

The Treatment of Colon Bacillus Infections of the Urinary Tract by Means of Subcutaneous and Intravesicle Injections of Bacteriophage Filtrates. Detailed Case Reports. Methods for Preparation of Filtrates

By D. Murray Cowie, M.D., and Wm. C. Hicks, Ann Arbor, Mich.

THE accompanying report records the treatment and progress of 46 cases of urinary tract infection with one or more strains of the colon bacillus and one with bacillus typhosus (Case 7). These records are arranged so as to make it easy for the reader to come to his own conclusions. There is much unwarranted enthusiasm concerning the use of the bacteriophage as a general measure in infections of all kinds, and in conditions that are secondary to them. Bacteriophage treatment has not yet reached the point where it is unnecessary to individualize. That there is an important place for the bacteriophage in therapeutics cannot be questioned. Its preparation and administration should be under the direction of competent medical men.

Great conservatism should be exercised in order to acquire as much trustworthy information as possible. No field in medicine is so full of variables as clinical medicine, particularly that side which has to do with therapeutics. With work of this character we have a great opportunity for the acquirement of fairly exact information, since technic can be so carefully executed and bacteriologic work so carefully controlled.

There has been no attempt to secure a large number of cases to report. Those that are recorded came in the ordinary run of our Clinic and in private practice. They represent, of course, only a fraction of those that come to the various clinics of the University Hospital. The wish has been that the cases might be studied carefully and for long periods of time. This has been possible in quite a number of them and will be recognized. The records unfortunately are not all as full and complete as they should be. This is due in part to State patients coming to the Hospital with no, or very inadequate, histories, to the necessity of dispensing with the services of special nurses, and to the discharge of these cases from the Hospital as soon as possible after the disappearance of symptoms and of the bacilluria. This has made it difficult to secure satisfactory follow-up notes in some cases. Hence it is only possible to say concerning these that sterilization was secured following bacteriophage treatment.

Several problems present themselves for consideration in the treatment of all colon bacillus infections of the urinary tract. One cannot know too much about the history. Have there been previous attacks? Have there been unexplained illnesses in the past, particularly if associated with fever, pains in the abdomen and back? Has there been a recent infection, an upper respiratory infection, for example, which may have been a predisposing factor? How long have the present symptoms or manifestations been present? In other words, are

*From the Department of Pediatrics and Infectious Diseases, University of Michigan Hospital and The Cowie Hospital.

we dealing with an acute, an acute recurrent, or a chronic infection? One must start out with the knowledge that these different types of infection do not all respond alike to treatment with the bacteriophage. On purely theoretical grounds we may expect a case of recent development to respond more promptly to treatment than a chronic one. In persistent cases a pyelogram should be made. If the case falls in the chronic group a guarded prognosis as to the duration of treatment and of observations should be given; some idea as to the prospects for ultimate sterilization of the urinary tract.

DEVELOPING OR ADAPTING THE BACTERIOPHAGE FILTRATE

Elsewhere we have described in detail the methods employed for the development of filtrates for treatment. After many years of diligent effort it has been impossible to develop a suitable polyvalent stock bacteriophage filtrate which could be recommended or dispensed for treatment of B. coli urinary tract infections in general. A potent filtrate for one patient's B. coli, which may have taken many weeks to develop, may have no lytic effect whatsoever on an organism isolated from another patient. On the other hand, it may so happen that a filtrate for the second patient may be developed from sewage base or from a stock phage in a few day's time.

All bacteriophage filtrates are polyvalent in the sense that they are lytic for more than one organism, particularly for old laboratory strains. It is always possible to enhance the potency of a bacteriophage from sewage base or from stock filtrates. Among stock filtrates kept on hand, which usually number nine or ten, we often find one that is satisfactory for treatment. If this happens many days' time may be saved.

The greatest problem of all is the adaptation of the bacteriophage to the case strain. The many illustrations of prompt sterilization after an entirely satisfactory bacteriophage has been found, inclines one to feel that no matter how long the infection has been running, if this can be achieved, a satisfactory result will occur. This is well illustrated in the following case:

CASE 30.—A. W. Bacteriophage adaptation was begun September 16, 1928. A partially potent bacteriophage was not obtained for twenty-four days. It was decided to try it out. After two months' treatment it was of no avail. The patient passed from observation for sixteen months, when she returned for further treatment (March 10, 1930). Because the original bacteriophage proved to be lytic for organisms of several other patients it was kept as a "stock phage." It had been constantly under development all this time, often being fed three times a day with this patient's organism. It was now found to have a very vigorous action against the patient's strain. Nine days after the first subcutaneous injection sterilization was complete. Fourteen subsequent urine cultures over a period of sixteen days were negative. Eleven months later a urine sample showed no cells. There had been no return of symptoms. Unfortunately the intern who saw the patient at this time failed to send a sample of the catheterized specimen to the laboratory.

CASE 29.—I. R. In this case the development of a satisfactory filtrate took two months' time. It was very potent for the patient's strain. In this patient no attempt was made to treat her with a partially lytic bacteriophage. Three days after the first subcutaneous injection the urine became sterile and continued so for five successive cultures over a period of eight days. There has been no recurrence.

It is true that there is more difficulty in finding a satisfactory bacteriophage for the long standing cases. On the other hand, one may be found in a comparatively short time, as illustrated in the following case:

CASE 27.—D. D., 5 years old, had had pyuria and bacilluria for over a year. A very potent filtrate was developed in ten days. The urine became sterile in three days after the first injection and remained so for three successive cultures over a period of four days, when she was discharged. A letter from the mother a month later reports gain in weight, marked general improvement, and absence of symptoms.

Experience teaches that some of these apparently resistant cases will recover if treatment is persisted in, rather if *laboratory efforts* are persisted in, long enough. That the bacteriophage benefits these long standing cases, even though we may be unable to entirely eliminate the organism, is shown by the clearing up of the clinical symptoms and the disappearance of pyuria under its use. In the case (Case 35) of longest duration, probably thirty years, foul urine, cloudiness and excess of cells disappeared.

When the acute cases, those of recent origin that do not give a history of previous attacks, are considered, we find that a satisfactory filtrate is secured in from one to twelve days. In nine it was secured within a week's time. In one case it took nineteen days to develop a suitable bacteriophage. It should be noted that for some unknown reason at this particular time difficulty had been experienced in promptly developing a filtrate for one or two other cases.

Under the influence of treatment with the lytic principle, it is a common observation to see one strain or colony type apparently changed to another. That is a sensitive type changed to a resistant type. In our use of the letters S and R we mean sensitive and resistant. We have not always been able to convince ourselves that all these resistant types produce rough colonies. The impression is easily gained that recurrences are due to the reappearance of the sensitive strain which is often a smooth colony producer. It seems quite probable that the sensitive type may develop from the resistant type which is often a rough colony producer. The greater number of colon bacillus infections of the urinary passages in our experience are of the single type.

Alkalinization.—If during the time bacteriophage development is going on the patient is being alkalinized, a certain amount of time is saved. Professor D'Herelle showed quite early in his work that the bacteriophage is not resistant to acid. It thrives best in slightly alkaline media (–6 to –12). "Lysis will not take place in a medium of acid reaction." "The bacteriophage ceases to grow when the medium presents the slightest acidity." Poncher and Cowie have verified these statements. They were able to develop a bacteriophage resistance to temperature above 75° C. but not to acidity.* These observations were of course done in vitro. Because of this fact alkalinization has been an essential procedure in the treatment of all of our cases. Our routine method at the present time is to put the patient on a basic diet and sufficient sodium bicarbonate alone or together with sodium citrate to render the majority of the urine samples alkaline. If during this treatment the urine occasionally becomes slightly acid to litmus, bacteriophage action seems to continue (examples: Cases 24 and 35). In those cases where careful observations were made on the urine reaction, we were convinced of the necessity of this procedure. We have had cases that showed no evidence of bacteriophage action while the urine remained acid, but which did after the urine became neutral or alkaline.

Alkalinization is not always an easy condition to produce unless one sets out to insure its presence. A blanket order for one patient may not fit another. It is best to give the patient pink litmus paper and instruct him how to test his urine. If during the time of phage development the patient is undergoing alkalinization, particularly if it is a long period, and if after the first one or two injections of bacteriophage the urine becomes sterile, one may wonder if the benefit came from the bacteriophage or the alkali. Most physicians have seen cases of B. coli infection of the urinary tract apparently recover on the use of alkali alone, so far at least as the disappearance of symptoms is concerned. This experience is by no means regular. The result following bacteriophage treatment is often so prompt and so marked as to leave no doubt in the observer's mind as to its efficacy. The effect in long standing cases, those that have resisted other methods of treatment including alkalinization, are of particular significance. They offer the greatest proof that the bacteriophage may be effective in the treatment of this type of infection. The almost regular sterilization of acute cases following injection of a bac-

*Unpublished record, 1923.

teriophage filtrate which we ourselves are entirely satisfied with convinces us that the bacteriophage is responsible. Sterilization in those cases in which the urine reaction has not been entirely satisfactory so far as alkalinity is concerned, where it has been slightly acid, is additional evidence in favor of the bacteriophage being responsible for the sterilization.

Autobacteriophage.—Because of the possibility, some might say the probability, of the development of an autobacteriophage in the patient's urine, it is important to utilize the principle of alkalinization. One of us has used alkalies in patients for other disturbances for many months at a time in doses commensurate with those we use in bacteriophagy without inducing untoward results. This is particularly true of certain gastrointestinal disturbances associated with hyperchlorhydria. We think no harm will come from this provided a case is a suitable one for alkalinization, and the alkali is given at the proper time in relation to the meal. So long as no edema, no unexplained increase in weight, and no breathing disturbance occur, it is safe.

We have not been successful in regularly demonstrating the bacteriophage in the urine of patients with B. coli infections either before or after bacteriophage injection. Others have done so, particularly after the injection of bacteriophage. They report that it is an almost invariable finding. We have, however, found three cases in which a bacteriophage had developed in the patient's urinary tract. These have been of great interest. In one (Case 16) it was discovered at the time of the first culture. It occurred in a patient who had been sent to Contagious Hospital because of a suspicion of typhoid fever. Many slowly motile gram-negative bacilli, which we believed were B. coli, were found in the urine. Blood culture and other typhoid tests were negative. The patient's blood had developed no agglutinins for B. typhosus. This boy of eight years, who had been ill for five days preceding entrance to the Hospital, became afebrile by lysis on the third day in the Hospital. The motile bacilli disappeared inside of twenty-four hours after this. They could not be cultivated. The urine contained a bacteriophage that was lytic for several other case strains and for a strain of B. Coli isolated from the patient's stool. This illustrates the characteristic manner in which a bacteriophage reaches a high state of potency just before the termination of an infectious process, and suggests that colon infections are naturally terminated this way and that it might be possible to find an active bacteriophage in the urine of such cases should we be able to follow them from day to day as we do a typhoid or a dysentery patient.

In another case (Case 44) which was brought to the Hospital because of a supposed acute appendicitis, and which was referred to this Department eight days later for study, a urine culture taken at this time (Feb. 20, 1931) was found to be loaded with colon Bacilli. Alkalinization was bgun. Three days later there were definitely fewer organisms in the urine, and an autobacteriophage was discovered. It was still present eight days later. It had little power against the organism it had been living with symbiotically. We endeavored to enhance its virulence artificially and succeeded in doing so to a certain extent. By the sixty-fifth day after the first injection of the enhanced autophage, the same organism could still be isolated from the patient's urine. When this culture was left alone, it lysed itself in forty-eight hours, making it impossible for us to keep a strain for further study. The urine was found to be sterile fifteen and twenty days later. No other bacteriophage was used in this case. In another case (Case 43) a bacteriophage appeared in the urine sixty-seven days after the last of four inoculations of bacteriophage filtrate.

Method of Administering the Bacteriophage.—There are three methods of administering bacteriophage filtrates, subcutaneous, intravesicle, and by direct ureter catheterization. All of these methods may be employed singly or combined as the case may indicate.

Subcutaneous Injection: Early experiments of D'Herelle indicate that small doses are definitely more effective than large ones. In his experimental work with Barbone which may be regarded as fundamental, a "solid immunity" could be conferred in forty to sixty days after a single injection of 20 c.c. of the bacteriophage filtrate, in twenty-eight days after 5 c.c., in twenty days after 0.25 c.c., and in four days after the injection of 0.04 c.c. If the animals were inoculated with a culture of barbone earlier than the intervals indicated for the respective amounts of specific bacteriophage, the animals died. Professor D'Herelle indicated the dose of 2 c.c. for the treatment of colon bacillus infections in man. We have used this amount, and

never more than 3 c.c. subcutaneously. It has been our custom to give the amount on alternating days for three doses.

In our first series we used only the subcutaneous method. There are 10 acute cases which were treated this way. The urine became sterile in 10, 17, 19, 15, 11, 7, 5, 4, 1, and 5 days respectively. There are nine acute cases treated by the combined subcutaneous and intravesicle methods, 3 c.c. in the arm, 8 to 10 c.c. instilled into the bladder. The urine became sterile in 8, 2, 2, 6, 2, and 4 days respectively in six, one (Case 16) developed an autophage, one (Case 12) could not return for culture. From this experience it would seem that the combined method is preferable. As the instillation of bacteriophage filtrate into the bladder produces no irritation it seems advisable to recommend this method of treatment for all cases. Should the infection be a cystitis alone, this type of treatment might prove more efficacious. We have treated only two cases by means of flushing the kidney pelvis with bacteriophage filtrate. No special advantage was gained over the other methods.

There are four acute recurrent attacks. In three cases treated by subcutaneous injection alone, (Cases 19, 20 and 22) the urine became sterile in 3, 3, 9, and 2 days respectively. One case (Case 21) treated by the combined arm and bladder method became sterile in two days. There are 10 attacks in 8 chronic cases treated by subcutaneous injection alone. The urine became sterile in 13, 3, 9, 3, 2, and 1 days respectively in 6 of these (75 per cent), not at all in 3, and in 11 months in one. There were 19 attacks in 12 cases treated by the combined arm and bladder method. The urine became sterile in 9, 1, 1, 7, 33, 33, 6, 5, 33, 15, 5, and 1 days respectively in 12 of these (63 per cent). In 5 attacks we were unable to sterilize the urine. The average number of days necessary for sterility by the subcutaneous method alone was 6, by the combined method 9. If we exclude the exceptionally long case (Case 35), the average number of days for the combined method was 6+. Again the combined method seems to be the better.

Bladder Instillation: The patient should present himself for treatment with a full bladder. This insures an adequate sample, and if a man, we think catheterization is easier. He has been instructed to reduce his water intake somewhat before treatment and to refrain from excessive drinking until five or six hours after instillation. The patient should lie down, preferably in bed. He is instructed to hold the urine as long as possible, the idea being to retain the bacteriophage as long as possible. Most people have no trouble in retaining the urine five or six hours. With children it may be more difficult. While this is not an absolute rule, we think it has certain advantages.

Reactions: In our experience, the severity of the reaction is in proportion to the amount of protein the filtrates contain. We have sometimes found that a bacteriophage developed in broth may have a higher potency than that developed on hard media. The latter contains the smaller amount of protein. Its solutions are practically water white. The potency of a bacteriophage may be developed so high in broth as to permit of very high dilution. Such filtrates may not produce marked reactions. These reactions are usually the well known nonspecific protein reactions, such as those that follow the subcutaneous injection of sterile milk or dead typhoid bacilli. If the patient becomes sensitized or has previously been sensitized to any of the proteins contained in the filtrate, a specific reaction may occur. The local and general reactions may be so severe that one may question the advisability of proceeding with the treatment until a more satisfactory filtrate is secured. As a rule, in the larger percentage of patients treated, no uncomfortable reactions occur. With the clear filtrate off solid media only a faint pink local reaction measuring 3 by 3 cm. to 5 by 5 cm. or less, with little or no edema, will be observed. A filtrate that will give a very marked reaction in one patient may have no effect whatsoever in another. No untoward results have followed any of the many hundreds of injections we have given. Children as a rule react less severely than adults.

The question may quite properly arise as to whether the protein content of the filtrate may not be responsible for the disappearance of the colon bacillus from the urine. Indeed we have seen bacteriophage filtrates that have been used in a somewhat promiscuous way in other types of infection which caused us to wonder if some of the effects produced may not have been due to the nonspecific action of the protein. Nonspecific protein therapy has been used

quite extensively in urinary infections. Cowie* has given this subject considerable consideration. He found that 3 c.c. of normal horse serum injected subcutaneously causes the urine in certain cases to become sterile. He had no success with the use of dead typhoid bacillus protein. Because of this fact a special effort has been made to keep the protein content of the bacteriophage filtrates as low as possible. A very convincing experience in favor of the bacteriophage being the responsible factor in addition to its obvious effect on the bacterium in vitro is the fact that sterilization by means of the bacteriophage is the same whether the protein content of the filtrate is high or very low, whether a reaction occurs or not. The case recorded in which a powerful autobacteriophage developed and which terminated in prompt recovery is a very good illustration that the bacteriophage alone was responsible for the cure.

Effect of Repeated Attempts to Sterilize the Urinary Tract with Bacteriophage Filtrate.—It has been feared because of the development of bacteriophage antibodies in a patient treated with bacteriophage filtrate that a second series of injections would be of no avail. This is probably due to a misunderstanding of the original experiments on bacteriophage antibodies. D'Herelle† has shown that the bacterium builds up antilysins against the bacteriophage and by so doing may become more resistant in a given preparation than it formerly was. This occurs if a bacterial suspension is inoculated with a weak bacteriophage. He explains on the same ground the failure of a bacteriophage of the same type to produce lysis when it is massively inoculated into a bacterial suspension. Under this condition the bacteria produce a large amount of antilysin, whereas if a very small amount of the same bacteriophage filtrate is inoculated into a bacterial suspension which has been highly diluted lysis is complete in twenty-four hours. Because of the high dilution the inhibitory substance has become inactive. This is particularly noticeable when transplants are made on solid media. In the treatment of B. coli infections this factor does not enter.

D'Herelle has also shown that antibodies of various types develop in the blood of rabbits following the injection of certain bacteriophage filtrates. It should be remembered that these filtrates contain not only the bacteriophage corpuscles but also the metabolites of the bacteriophage action on the bacteria. Thus there are produced amboceptor, agglutinin and opsonic antibodies against the bacterium and antitoxin and antiferments against the secretory products of the bacteria. In the case of B. coli he found bacterial antibodies, agglutinins, for certain strains. These are "usually so weak that their action is almost imperceptible." He is of the opinion that the presence of bacterial antibodies in the blood are of no special significance. The situation is not analogous to that of antibacteriophage Shiga dysentery serum. The Shiga bacillus develops an endotoxin which in turn develops an antitoxin and antibacteriophage antibodies that sensitize the Shiga bacillus and render it more virulent for mice. In this instance an antiimmunizing serum is contained in the bacteriophage antiserum. D'Herelle points out that this sensitizing substance, sensibilisine, "develops in the animal only after the second injection," and "but a single minute injection of a culture of antidysentery bacteriophage" is necessary effectively to vaccinate a rabbit against the effects of the toxin.

When this knowledge has been applied to man, it has been found that the injection of sensitized bacteria are of no greater virulence in general than normal living organisms. "They are equivalent." From records of cases contained in this report it will be readily seen that one course of one series of injections of the bacteriophage filtrate does not interfere with a subsequent series producing lysis of B. coli in the urinary passages whether given at short or long intervals.

No bacteriophagocidal antibodies develop in an individual after inoculation with bacteriophage filtrate. Mrs. J. O., Case 23, received in all, six injections of filtrate in two series. If phagocidal antibodies develop and are of any significance, one would expect to find them in this patient's serum. With the assistance of Mr. Robert Hicks, various amounts of bacteriophage were subjected to the action of this serum for several hours at 37° C. Even in those tubes that contained only minute amounts of the bacteriophage and a high concentration of serum, no difficulty was encountered in recovering a bacteriophage as vigorous in its action on B. coli as it was before being acted upon by the patient's serum.

---

*The Horse Serum (Foreign Protein) Treatment of Pyelitis and Pyuria, Am. J. Dis. Child. **24**: 179, 1922.

†D'Herelle: The Bacteriophage, Eng. Trans., 1922, Williams & Wilkins Co.

Effect of Bacteriophage Treatment on the Cell Content of the Urine.—Usually the disappearance of pus or increased cells in the urine precedes or occurs simultaneously with the disappearance of B. coli. In 38 cases, the excess of cells disappeared before the urine became sterile in 19, simultaneously in 16, and after it became sterile in 3. The disappearance of cells or the subsidence of all symptoms, however, cannot be taken as a criterion for the disappearance of B. coli. This will be seen by consulting the records. Unfortunately in some cases notes on the cells are inadequate. This was due to a centering of interest on the bacteriology. A perusal of the cases will convince one that the treatment as carried out has in most cases a definite effect on the pyuria. On the other hand, almost invariably when bacilluria is completely overcome pyuria does not continue.

Discharging the Patient: All patients on discharge are requested to continue the alkalinization under the direction of their home physician or the Clinic. Those that are apparently cured are informed that the condition recurs in some cases. They are asked to report if any manifestations occur, and are advised that the earlier they are attended to the easier it is to overcome the infection.

ACUTE COLON BACILLUS INFECTIONS, 18 CASES

The criteria for the diagnosis of acute colon bacillus infections of the urinary tract in addition to the demonstration of the colon bacillus are: (1) the recent occurrence, that is, within a few days to a few weeks, of the well known acute manifestations; (2) the occurrence of manifestations referable to the urinary passages during the course of some other disease, usually an infectious disease; (3) the occurrence of manifestations following a surgical operation, particularly abdominal and pelvic, and following catheterization.

TABLE I

CASE	AGE	SEX	APPROXIMATE DURATION BEFORE TREATMENT WITH BACTERIOPHAGE	URINE BECAME STERILE AFTER BEGINNING TREATMENT IN	NUMBER OF TREATMENTS	URINE REMAINED STERILE FOR AT LEAST*	RETURN OF BACILLURIA AFTER	METHOD OF ADMINISTRATION	DATE OF ENTRANCE
			DAYS WEEKS						
1	10 m	F	3+	10 days	3	10 days	13 days	Subcutaneously	6-4-25
2	12 yr.	F	12	17 days	3	10 days	No return	Subcutaneously	6-13-25
3	4	F	5±	19 days	6	8 days	No return	Subcutaneously	6-15-25
4	27	F	7	15 days	4	5 days	No return	Subcutaneously K	6-28-25
5	Ad.	F	2+	11 days	4	9 days	No return	Subcutaneously	9-22-25
6	33	F	4+	7 days	3	5 days	No return	Subcutaneously	10-15-25
7		M	Typhoid	5 days	2		No return	Subcutaneously	10-26-25
8**	50	F	3		3		No return	Subcutaneously	6-25-28
9	66	F	6	1 day	3	6 days	No return	Subcutaneously	11-17-28
10	52	F	2+	5 days	3	50 days	No return	Subcutaneously	5-16-29
11	55	F	2+	8 days	8	12 days	No return	Subcutaneously B	12-29-30
12**	45	F	2		3		No return	Subcutaneously B	2-27-30
13	50	F	2	2 days	4	20 days	No return	Subcutaneously B	2-10-31
14	3	F		2 days	3	2 days	No return	Subcutaneously B	6-23-31
15	9	F	6	6 days	3	2 days	No return	Subcutaneously B	10-2-31
16***	8	M	4	1 day Autophage			No return		11-18-31
17	6	F	7	2 days	3	2 days	No return	Subcutaneously B	12-10-31
18	7	F	10	4 days	3	1 day	No return		12-16-31

K—Kidney pelvis lavage. B—Bladder.

*Last opportunity for a culture.
**Could not return for check culture. Symptoms disappeared. No recurrence to date.
***An autobacteriophage was found in urine at first culture. Urine sterile following day. See record.

There are 12 cases belonging in the first group (Cases 2, 4, 5, 6, 10, 12, 13, 14, 15, 16, 17, 18) (see Table I). The urine became sterile in two to seven days in all but the first three, which required 17, 15 and 11 days respectively. Case 12 we assume became sterile. The patient has been under regular observation. There has been no recurrence to January, 1932. There are three cases in the second group (Cases 1, 3, and 11). The urine became sterile after a longer period, 10, 19, and 8 days respectively. In Case 1 there was a reappearance of B. coli after thirteen days. Twenty-two days after a second course of three subcutaneous injections, the urine remained sterile for thirteen days. In the last group there are two cases (Cases 8 and 9). One responded with a negative culture in twenty hours and continued to be negative. The other responded with disappearance of symptoms and cells and a distinct diminution in the number of bacteria. There was no opportunity for further culturing. The patient had no return of symptoms. It may be permissible to say this case was at least clinically cured.

There are not enough cases in the last two groups to warrant any conclusion as to a difference in time for sterilization to occur. The analysis suggests that the earlier the case is placed under treatment the more rapidly it will clear up.

CASE 1.—N. R., aged ten months. U. H.* 10536. On May 14, 1925 the baby was brought to the Hospital because of hydrocephalus, spina bifida and clubbed feet. Two days after

Case 1. Counted as two attacks of bacilluria.

DATE 1925	CELLS*	B. COLI	URINE P. H.	REMARKS
6-4	+	+**	6.1 acid	Alkalinization begun
5	++		6.2 acid	
6		+	6.9 acid	3 c.c. bacteriophage filtrate in arm
8		+	7.2 alk.	2 c.c. bacteriophage filtrate in arm
10				2 c.c. bacteriophage filtrate in arm
13	0	+	7.1 alk.	
16	0	0	7.2 alk.	
17	0	0	7.2 alk.	
18	+++	0	6.5 acid	
25	0	0		Gaining weight
28	0			
7-7	++	+	6.5 acid	
9	++	+	6.9 acid	3 c.c. bacteriophage filtrate in arm
12		+		2 c.c. bacteriophage filtrate in arm
15				2 c.c. bacteriophage filtrate in arm
31	0	0	7.3 alk.	
8-1	0	0	7.4 alk.	
8-5	0	0	7.4 alk.	
12	0	0	7.4 alk.	

*Per low power field uncentrifuged urine.

***Estimation of the degree of positiveness!* In all of the 1925 observations the cultures were marked + or 0 without any attempt to indicate the numbers of bacilli. In subsequent reports we have adopted the following scheme. Inoculations are made with a standard 2 mm. platinum loop. ++++, growth completely covering a 3-inch Endos Petri dish in twenty-four hours; +++, about 200 colonies; ++, 50 to 75 colonies; +, about five colonies; ±, no growth on plates but an organism can be developed in broth with 3 c.c. of urine; 0, no growth in broth with 3 c.c. of urine. Many reporters regard a negative Endos Petri dish as evidence of sterility. Three cubic centimeters of urine in broth is a much more rigid test. Final negative sterility must be on media other than Endos (blood agar or broth). It is of interest to note that if counts are below fifty they are usually about five.

*U. H., University Hospital, C. H., Cowie Hospital.

entering, a temperature of 103.5° developed. Symptoms of an upper respiratory infection were present. On May 22 the urine showed pus and a trace of albumin. On June 4 a urine culture showed B. coli. *Diagnosis:* upper respiratory infection complicated by urinary tract infection, probably pyelitis. Alkalinization was begun. A satisfactory bacteriophage was found in stock. Three subcutaneous treatments on alternate days brought about a termination of the symptoms.

Recurrence of cells and B. coli July 7 indicated the necessity for a second series of injections. The pyuria and bacilluria disappeared in twenty-two days, and continued for thirteen days. No recurrence.

CASE 2.—C. V., aged twelve. U. H. 128816. On the morning of June 10, 1925 the patient complained of pain in the right lower abdomen and a feeling of malaise. The following morning she vomited. The temperature was 104°, where it remained until admittance to the Hospital June 13. She vomited several times after entrance. There was no history of a previous attack. There had been frequency and burning micturition. The patient was sent to the Hospital with a diagnosis of acute appendicitis.

Physical examination showed a well developed, well nourished, acutely ill child, definite tenderness on pressure in the right lower quadrant of the abdomen and in the right costovertebral angle. Otherwise examination was negative. Leucocytes were 15,300. A catheterized urine sample was turbid and acid; albumin was positive. There were 25 cells per low power uncentrifuged field, some clumps. Culture showed B. coli.

Alkalinization and forced fluids were begun. The temperature reached normal in forty-eight hours, where it remained for several days. The morning of June 21 chill, temperature of 104°, pain in left lower abdomen, and marked tenderness in the left costovertebral angle developed. Five thousand cubic centimeters of fluid were given during the following twenty-four hours. The temperature dropped to 101.5. It will be observed that the so-called sensitive or lytic colonies disappeared from the urine twenty-four hours after the third dose of bacteriophage, the resistant colonies fourteen days after. The patient made a good recovery without recurrence. Detailed record follows.

CASE 2

DATE	CELLS	B.COLI R*	B.COLI L*	URINE P. H.	REMARKS
1925					
6-13	++	+	+	5.8 acid	Temp. 104°. Alkalinization begun
6-16	++	+	+	7.3 alk.	Temp. 99°
6-19	++	+	+	7.3 alk.	Temp. 98.4°
6-20	+	+	+	7.2 alk.	Temp. 100°
6-21	++	+	+	7.3 alk.	Chill, temp. 104°, pain 1.1. abdomen and 1.0 costovertebral angle
6-22	++	+	+	7.2 alk.	3 c.c. bacteriophage filtrate in arm. Temp. 104° continued
6-23		+	+	7.2 alk.	Temp. 98.5°
6-24		+	+	7.4 alk.	2 c.c. bacteriophage filtrate in arm
6-25	++	+	0	7.2 alk.	24 hours after last injection
6-26		+	0	7.4 alk.	2 c.c. bacteriophage filtrate in arm
6-28	+	+	0	7.2 alk.	
6-30	+++	+	0	6.8 acid	Temperature remained normal. No symptoms
7-2	++	+	0	6.5 acid	
7-4	++	+	0	6.8 acid	
7-5	+				
7-7	+	+	0	6.6 acid	
7-9	±	0	0	6.8 acid	14 days after last injection
7-11	±	0	0	6.8 acid	
7-12	±	0	0	6.8 acid	
7-13	0	0	0	6.8 acid	Temperature normal for 15 days
7-15	0	0	0	6.9 alk.	
7-16	0	0	0	7.2 alk.	
7-17	0	0	0	7.1 alk.	
7-19	0	0	0	7.1 alk.	No recurrence of symptoms

*R—resistant; L—lytic.

CASE 3.—D. P., aged four. U. H. 131261. This patient was in the Hospital for over a year because of an extensive burn. On June 1, 1925 she was sent to Contagious Hospital because of measles. On June 15 she was transferred back to the Children's Ward. During the routine urine examination, pus and many motile bacilli were found. Alkalinization was started. A bacteriophage filtrate was ready for injection June 19. Two courses of filtrate were necessary to clear up this case. Thirteen days after the last injection the urine became sterile and remained so.

CASE 3

DATE	CELLS	B. COLI	URINE P. H.	REMARKS
1925				
6-15	++++	+	6.2 acid	Alkalinization begun
6-16	++++		6.3 acid	
6-17	++++	+	6.3 acid	
6-18	++++	+	6.3 acid	
6-22	++++	+	6.2 acid	
6-23	++++	+	6.3 acid	2 c.c. bacteriophage filtrate in arm
6-24				2 c.c. bacteriophage filtrate in arm
6-25				1 c.c. bacteriophage filtrate in arm
6-28		+		
7-1	++		7.4 alk.	
7-2	0		7.4 alk.	
7-3	++	+	7.4 alk.	3 c.c. bacteriophage filtrate in arm
7-9	++	+	7.4 alk.	3 c.c. bacteriophage filtrate in arm
7-13	+++		6.9 acid	2 c.c. bacteriophage filtrate in arm
7-15	+++		6.9 acid	2 c.c. bacteriophage filtrate in arm
7-16	++++			
7-18	++			
7-22		+	7.1 alk.	
7-28	0	0	7.2 alk.	
7-30	0	0	7.2 alk.	
8-1	0	0	6.9 acid	
8-5	0	0	6.9 acid	

CASE 4.—Mrs. A. Q., aged twenty-seven. U. H. 122366. The patient came to the Hospital June 28, 1925 because of pain in the left side and back. About one week before entrance she noticed "bladder trouble," as evidenced by marked frequency, nocturia, and burning. Urinary frequency became almost constant, and pain became continuous in left side and back. However, the pain was worse during certain attacks. She never passed blood; no vaginal discharge. Temperature rose progressively and her doctor found much albumin and pus in the urine. She was given hexylresorcinal treatment. Sinusitis of the left antrum three months preceding admission was reported. She was ill five weeks. Patient was pregnant.

CASE 4

DATE	CELLS	B. COLI	URINE P. H.	REMARKS
1925				
6-28	+++	+	5.9 acid	Right ureter B. coli +, left 0. Alkalinization begun
6-29		+	5.8 acid	
7-1	++	+	7.4 alk.	3 c.c. filtrate in arm, 25 c.c. pelvic lavage
7-4	+	+	7.3 alk.	By mistake hexylresorcinal was not discontinued June 28, 1925
				2 c.c. filtrate in arm
7-5	++	+	7.4 alk.	
7-8	+++			2 c.c. filtrate in arm
7-9		+	7.6 alk.	
7-10		+	7.6 alk.	2 c.c. filtrate in arm
7-14		+	7.2 alk.	
7-15		0	7.2 alk.	
7-16		0	7.2 alk.	
7-20		0	6.9 acid	Condition has cleared up satisfactorily

Physical examination: Temperature 104°. Otherwise negative. On July 1, 1925 cystoscopic examination was done. Both pelves were lavaged with bacteriophage solution.

This patient was given four subcutaneous injections of bacteriophage solution. The urine became sterile on the eleventh day after the first dose. Whether hexylresorcinal delayed sterilization or not cannot be determined. Urinary antiseptics should not be administered during treatment with the bacteriophage.

CASE 5.—Mrs. O'B., aged forty-seven. C. H.,* September 22, 1925. The patient complained of painful micturition for over two weeks. There were no other symptoms. She was otherwise in excellent health. The urine showed a marked excess of leucocytes. A catheterized specimen confirmed this finding and was positive for B. coli. Alkalinization was begun and was effected in three days. Sterilization occurred in eleven days. The patient's symptoms were definitely improved after the second treatment, and had disappeared by the eleventh day. There has been no return of symptoms in this patient.

CASE 5

DATE	CELLS	B. COLI	URINE P. H.	REMARKS
1925				
9-23	++++	+	5.8 acid	
9-25				3 c.c. filtrate in arm
9-26	++++	+	7.6 alk.	
9-27				2 c.c. filtrate in arm
9-28	++++	+	7.6 alk.	
9-29				2 c.c. filtrate in arm
10-1	++++	+	7.6 alk.	
10-2		+	7.8 alk.	2 c.c. filtrate in arm
10-3	+	+	7.4 alk.	Colonies changing in character
10-6	±	0	7.5 alk.	Symptoms have entirely disappeared
10-10	0	0	7.5 alk.	
10-15	0	0	7.4 alk.	No return of symptoms

CASE 6.—Mrs. G. W. I., aged thirty-three. C. H., October 15, 1925. The patient had chronic nephritis with hypertension for two years. A few days previous to October 15 she developed urethral irritation. A catheterized urine specimen showed 100 to 150 cells per low power field. Culture showed B. coli, type R, large white convex homogeneous colonies and type S, small flat gray translucent colonies. In four days we adapted a bacteriophage that was lytic for both types. The urine became sterile seven days after the first treatment.

CASE 6

DATE	B. COLI R	B. COLI S	URINE P. H.	REMARKS
1925				
10-15	+	+	6.2 acid	Alkalinization begun
10-19				3 c.c. filtrate in arm
10-20	+	+	7.4 alk	
10-21				3 c.c. filtrate in arm
10-23	0	+	7.4 alk.	3 c.c. filtrate in arm
10-26	0	0	7.6 alk.	Symptoms have disappeared
10-31	0	0	7.6 alk.	

CASE 7.—Mr. L. P., aged twenty-eight. U. H. 127787. October 26, 1925. The patient entered Contagious Hospital October 12, 1925 with typhoid fever. The course of the disease was uneventful. The urine culture continued to be positive for B. typhosus preventing his discharge. Cultures on October 26, 28, 30, and November 4, 6, 8, and 10 were positive. Urine alkalinization was effected, and 3 c.c. of bacteriophage filtrate were injected sub-

*C. H., Cowie Hospital. U. H., University Hospital.

cutaneously on November 6 and 8. On November 10 the urine culture was still positive. On November 11 culture was negative. There was no recurrence. This case is included in this group because of its general interest and the colon typhoid group of bacteria being so closely related with regard to the action of the intestinal bacteriophage.

CASE 8.—Miss G. Y., aged fifty. C. H. June 25, 1928. Cholecystectomy with drainage for cholelithiasis and acute cholecystitis.

June 27, 1928. Urine specimen showed sugar and three or four casts per field. Later in the day the sugar was negative, but the specimen was loaded with casts.

June 29, 1928. Sugar negative, no casts, marked excess of leucocytes.

July 4, 1928. Patient complained of irritation and frequency. Catheterized specimen showed cells ++++ and many bacteria.

CASE 8

DATE	CELLS	B. COLI	REMARKS
1928			
7-17	+++		Alkalinization begun
7-18	++++	++++	Large colonies
7-23		++++	3 c.c. filtrate in arm
		±	1 hour after treatment
7-24	±	++++	3 c.c. filtrate in arm
7-25		++	Tiny colonies. No phage in urine
7-26	±	++	3 c.c. filtrate in arm. No phage in urine
7-27	0	++	Symptoms have disappeared. No phage found in urine Colonies remain small type

Patient was discharged symptomless but still showing B. coli. She had no further trouble.

CASE 9.—Mrs. L. P. H., aged sixty-six. C. H. September 21, 1928. Cholecystectomy for cholelithiasis and cholecystitis, following a sudden attack of abdominal pain, fever, leucocytosis of 20,000, and evidence of empyema of the gall bladder on July 13, 1928. This infection was of over two weeks' duration. On November 7, 1928, forty-seven days after operation, the patient complained of frequency and urethral irritation. Pyuria developed. Urine culture showed ++++ B. coli. Alkalinization was begun.

CASE 9

DATE	CELLS	B. COLI	REMARKS
1928			
11-7	++++	++++	Alkalinization begun
11-13	++++	++++	3 c.c. filtrate in arm
11-14	0	0	20 hours after treatment
11-15	0		3 c.c. filtrate in arm
11-17	0	0	3 c.c. filtrate in arm
11-19	0	0	No phage in urine

The symptoms disappeared the day after the first treatment, and the urine became sterile in twenty hours.

CASE 10.—Mrs. V. C. W., aged fifty-two. C. H. May 16, 1929. The patient suddenly developed marked hematuria on May 15, 1929 without any apparent cause. She had been in excellent health. There was no history of a recent infection. Physical examination was negative. The first morning urine sample was dark red. There had been no pain on urination. Blood pressure 160/85. Nonprotein nitrogen 42, urine concentration normal, albumin +++, leucocytes and red cells +++. No casts. Alkalinization was begun. A satisfactory bacteriophage was developed in three days. The urine became sterile in five days, and free of organic elements. This patient had no subjective symptoms. There has been no recurrence.

CASE 10

DATE	CELLS	B. COLI	REMARKS
1929			
5-16	++++		Urine bloody
5-17	++++		Urine beginning to clear of blood. Alkalinization begun
5-18			Urine clear
5-24	++++		No casts
5-27	++++	+++	Bacteriophage adaptation begun
5-30	++++		No casts
5-31			3 c.c. filtrate in arm
6-1		+++	Small type B. coli. No phage demonstrated in urine
6-2			3 c.c. filtrate in arm
6-3		+	
6-4			3 c.c. filtrate in arm
6-5	0	0	
6-6			Discharged. Urine clear
6-10	0	0	
6-17	0	0	
6-25	0	0	
7-12	0		
7-30	0	0	

CASE 11.—Mrs. V., aged fifty-five. C. H. January 13, 1930. The day after Christmas the patient developed an upper respiratory infection. She was in bed five days. Had chills all this time. Five days later, on the tenth day, she developed urinary frequency, irritation, and pain on micturition. She had a similar attack six years previous following an attack of influenza. The urine on January 13 showed ++++ cells, many large clumps, and ++++ B. coli. Alkalinization begun.

CASE 11

DATE	CELLS	B. COLI	REMARKS
1930			
1-13	++++	++++	
1-14			3 c.c. filtrate in arm, 10 c.c. in bladder
1-16	+	±	3 c.c. filtrate in arm, 10 c.c. in bladder
1-18	0	+	3 c.c. filtrate in arm, 10 c.c. in bladder
1-20	±	++	3 c.c. filtrate in arm, 10 c.c in bladder
1-22	0	0	3 c.c. filtrate in arm, 10 c.c. in bladder
1-27	0	0	3 c.c. filtrate in arm, 10 c.c. in bladder
2-4	0	0	3 c.c. filtrate in arm, 10 c.c. in bladder
2-11	0	0	3 c.c. filtrate in arm, 10 c.c. in bladder
11-3	0	0	

A bacteriophage was adapted for the patient's organism in twenty-four hours by feeding it three times a day with the patient's organism. The urinary symptoms disappeared very quickly, and the urine became sterile eight days after the first injection.

CASE 12.—Mrs. D., aged forty-five. C. H. February 27, 1930. The patient had recently been having bladder irritation, frequency, and burning micturition. She was a robust, un-

CASE 12

DATE	CELLS	B. COLI	REMARKS
1930			
3-6	+	++++	3 c.c. stock filtrate in arm, 10 c.c. in bladder
3-8	±	++	3 c.c. stock filtrate in arm, 10 c.c. in bladder
3-9	++	++	
3-10	0	++	3 c.c. stock filtrate in arm, 10 c.c. in bladder

usually healthy individual. These symptoms were preceded some days previous by a severe leucorrhea, that developed on a long automobile trip, which made normal bladder emptying impossible. A catheterized specimen showed a few cells and ++++ B. coli. A suitable bacteriophage was not developed until the 8th day. During this time the urine became alkaline on the usual treatment.

The patient's symptoms were entirely relieved by the three treatments, and the excess of cells disappeared from the urine. It was not possible to make further cultures. There has been no recurrence to date. At least a clinical cure was effected in this patient.

CASE 13.—Mrs. P. B. S., aged fifty. C. H. February 10, 1931. Two weeks ago the patient began to have smarting and frequent urination. A sample of urine was said to have shown pus. The bladder was irrigated twice. This caused much pain. The past three or four days she has complained of general aching of the body, and a feeling of heat and chilliness. Gave no history of fever.

General examination aside from the urinary findings was negative. A catheterized urine specimen showed 10 to 15 cells per low power field, uncentrifuged urine, a slight excess of epithelial cells, no casts or red cells. She had been drinking large amounts of water. Urine culture on this date showed B. coli ++. Alkalinization was begun.

A satisfactory filtrate was developed from sewage base in ten days. It will be observed from the clinical record that no change had been effected during the first thirteen days except that the complaints had been improved. Two days after the first bacteriophage treatment the urine became cell and bacteria free. No recurrence to date.

CASE 13

DATE	CELLS	B. COLI	CLINICAL FINDINGS
1931			
2-10	+	++	Alkalinization begun
2-23	+	+++	Symptoms better. 3 c.c. filtrate in arm, 10 c.c. in bladder.
2-25	0	0	Symptoms disappeared. 3 c.c. filtrate in arm, 10 c.c. in bladder
2-27	0	0	No symptoms. 3 c.c. filtrate in arm, 10 c.c. in bladder
3-4	0	0	3 c.c. filtrate in arm, 10 c.c. in bladder
3-17	0	0	Continues to be symptom free. Urine alkaline Discharged. No recurrence to date

CASE 14.—M. G., aged three. U. H. 267842. June 23, 1931. Patient sent to Hospital because of a heart murmur, unconscious attacks associated with clonic spasms, and pus in the urine on several occasions.

Examination: Well nourished girl. Temperature 99°. No pathologic findings. Heart has been entirely negative. Kahn negative. Urine showed 500 cells per low power field. Eyes and fundi negative. Skull x-ray negative.

Diagnosis: Epilepsy, colon infection of genitourinary tract. This patient was not on a ketogenic diet.

CASE 14

DATE	CELLS	B. COLI	REACTION	CLINICAL NOTES
1931				
6-25	++	++++	acid	Alkalinization begun
7-2	++	++++	acid	
7-15	++	+++	alk.	3 c.c. filtrate in arm, 7 c.c. in bladder
7-16				Urine clear
7-17	0	0	neut.	3 c.c. filtrate in arm, 7 c.c. in bladder
7-19	0	0	alk.	3 c.c. filtrate in arm, 7 c.c. in bladder
7-24				Discharged. Urine clear. Return in six weeks

CASE 15.—D. S., aged nine. U. H. 275513. October 2, 1931. Patient came in with a history of attacks of fever beginning six weeks ago, accompanied by painful micturition, small clots of blood in the urine, costovertebral pain on both sides, especially on the right, loss of weight and headaches. The diet had been restricted as a remedial measure. The first attack lasted four or five days. The attacks recurred every four or five days. After the first attack they lasted from one to three days. The mother observed that these differed from the first attack in that with the subsidence of the attacks the urine became cloudy, while during the attacks it was clear. She has been confined to bed all this time.

Physical examination: Poorly nourished child. A small round smooth firm movable mass in the abdomen beneath the right costal margin is plainly felt, probably a kidney. She has complained of pain in this area. The lower pole of the left kidney is just palpable. Otherwise examination is negative. Temperature 99° to 100°, pulse 80 to 105, respirations 20 to 25.

Urine: Acid, trace of albumin, loaded with leucocytes and motile bacteria, a few red cells, no casts. Culture started October 2 showed B. coli ++++. Blood: hemoglobin 55 per cent, red cells 2,500,000, white cells 8,450, nonprotein nitrogen 31. Kahn negative.

Skiadan x-ray showed no evidence of organic change in genitourinary tract.

A satisfactory bacteriophage filtrate was developed in ten days. The urine became sterile on the fifth day after beginning treatment. It should be observed that this patient's urine showed marked improvement as indicated by a marked decrease in the number of cells and bacteria under the influence of alkalinization before the bacteriophage filtrate injections were given. It was not possible to keep the patient under observation longer. When last heard from there had been no return of the symptoms.

CASE 15

DATE	CELLS	B. COLI	CLINICAL RECORD
1931			
10-2	+		Alkalinization begun
10-3	+		
10-7	±	++	
10-14	++++		
10-15	+++	++++	Urine alkaline
10-17	+	++++	
10-22	0	+	3 c.c. filtrate in arm, 7 c.c. in bladder
10-24	±	+	Morning sample
	0	±	P.M. 3 c.c. filtrate in arm, 7 c.c. in bladder
10-26	±	±	Urine continues to be alkaline
10-27	0	±	Urine alkaline. 3 c.c. filtrate in arm, 7 c.c. in bladder
10-28	0	0	Discharged on sodium bicarbonate
12-10	0	0	Patient has remained perfectly well

One may question whether the alkalinization alone or the bacteriophage was responsible for the change. Our experience leads us to believe it was the bacteriophage.

CASE 16.—E. C., aged eight. U. H. 278667. November 18, 1931. Patient entered Contagious Hospital with a history of malaise which had been present for five days. A month before entrance he had had a diarrhea for a period of three weeks. He recovered from this and returned to school. Three days before admittance he complained of headache. He drank from a well of questionable water six weeks ago. His appetite failed, and he had an attack suggestive of a convulsion followed by coma November 17. He vomited once and complained of pain in the abdomen.

November 18, 1931. Temperature 104°, pulse 132, respirations 25. Leucocytes 16,700. Urine showed 4 to 8 leucocytes per low power field, a few red cells, an occasional cast, many epithelial cells, and many slowly motile bacilli which were gram-negative. Diazo reaction on the urine negative. Spinal fluid 3 to 5 cells, colloidal gold, mastic and Kahn negative. Blood serum Kahn negative. Blood culture negative.

This patient made an uneventful recovery. His temperature, pulse, and respirations returned to normal by lysis on the third day, and the motile bacilli disappeared from his urine inside of twenty-four hours, leading us to believe the case to be one of acute pyelitis.

Bacteriology: The bacteriology of this case is of considerable interest. The catheterized specimen showed many slowly motile bacilli. The sediment was crowded with them. On staining they were all gram-negative, characteristic of B. coli. The centrifuged sediment was 1¼ inches deep. The following morning no bacilli could be demonstrated.

The uncentrifuged urine and the sediment were cultured on plain broth, blood agar and Endos medium. The media had all been freshly prepared the day before. These preparations were placed in the incubator in the evening. The following morning there was no growth on the plates or in the broth. They remained negative for five days, at which time they were discarded.

After the above cultures were started, samples of the same urine and urine sediment were left at room temperature for twelve hours. We were interested to find that no organisms could be demonstrated in either specimen. Their previous presence had been checked by three of us.

We then tested the lytic power of the urine and sediment against four strains of B. coli, including a mixed colon culture from the patient's stool. These were planted on plain agar, dried, and carefully washed with urine, leaving control areas around the edges of the Petri dish. No growth occurred after three days. No secondary colonies have since developed. The control areas showed abundant growth.

November 24, 1931. Six days after entrance the urine of November 18, 1931, which had been kept in the refrigerator, was still clear. No organisms could be demonstrated. On November 18 and 19 an active bacteriophage was demonstrated in this urine.

Comments.—It would seem that this experience demonstrates very beautifully the phenomenon, shown by Professor D'Herelle, of the appearance of a bacteriophage of high potency just before the termination of the infectious process. The patient made a perfect recovery from this illness. Had we been one day later in making our observations a satisfactory diagnosis of the case could not have been made. It suggests the advisability of performing urine lytic tests on suspected cases in which a negative urine culture is obtained.

CASE 17.—R. M. K., aged six. U. H. 279952. December 10, 1931. The patient has complained of pain in the lower right abdomen for one week. Nausea and vomiting, fever and drowsiness occurred on the second day. Since this time, the pain has been irregular in its occurrence. With each recurrence of pain she vomits. No vomiting on day of admittance to Hospital. There was slight abdominal pain, no urinary symptoms. Temperature 101.3°, pulse 120. Patient looked acutely ill, cheeks were flushed. There was slightly increased muscle tonicity right lower abdomen, and some tenderness on pressure. No mass felt. Rectal examination negative. Patient cried when rectum was pressed anteriorly in the midline. Otherwise examination entirely negative. Leucocytes 16,000. Urine showed pus and many motile bacilli. Culture done December 10, 1931 showed ++++ B. coli. When informed of the diagnosis, the mother told us she had had "pyelitis" before. A stock bacteriophage filtrate was found to be very potent for the patient's strain. The urine became sterile within forty-eight hours after the first treatment, and has remained so. Alkalinization was begun on December 11, 1931. Abdominal pain and tenderness disappeared on the second day in the Hospital.

CASE 17

DATE	CELLS	B. COLI	REMARKS
1931			
12-12	+++	++++	Urine neutral. Stock bacteriophage developed potency in 5 days
12-17	+++	++++	Urine alkaline. 3 c.c. filtrate in arm, 8 c.c. in bladder
12-19	0	0	Urine alkaline. 3 c.c. filtrate in arm, 8 c.c. in bladder
12-21	0	0	Urine alkaline. 3 c.c. filtrate in arm, 8 c.c. in bladder

After the first injection the temperature reached 103° and the arm was sore. After the second injection the temperature was 102°. The arm was more swollen and painful. After the third injection the temperature reached 104° in four hours. The arm was not sore.

Bacteriology.—One of our stock bacteriophage filtrates was found to be very potent for this patient's strain of B. coli. It contained two plaques. The base for the small plaque bacteriophage was sewer water. It had been developed to a high potency against A.B.C. B. coli (Case 35). This was added to another stock bacteriophage secured from the urine of I. H. (Case 16) (an autophage), which was made up of large plaques. We were never able to demonstrate small plaques in this patient's urine. In this mixed bacteriophage filtrate the large plaque was very active against the patient's strain. The small plaques had little or no activity. (See notes on Case 18.)

CASE 18.—B. P., aged seven. U. H. December 16, 1931. Patient complained of pain in the "right hip" December 6. There had been fever, but no nausea, vomiting or chills, and no urinary symptoms. Temperature on day of admittance was normal, pulse normal, appetite fair. The pain was located in the abdomen just above the right anterior superior spine. There was no costovertebral pain or tenderness. There was a slight hemoglobinemia. The ordinary urine specimen showed pus and bacteria. Otherwise the examination was negative.

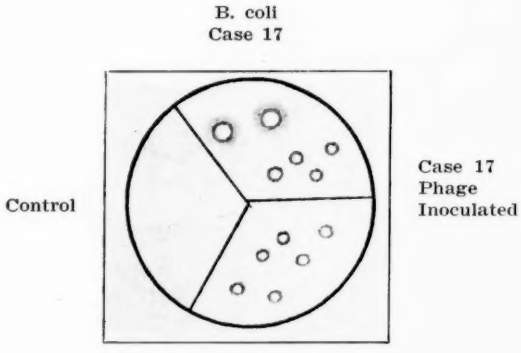

Fig. 1

The same bacteriophage as that used in Case 17 was very potent for this patient's B. coli. The large plaque bacteriophage of this filtrate had no action whatsoever; the small plaque was very active. This is a matter of interest in bacteriophage development and adaptation. This information is secured in the following manner. A Petri dish is divided into three sections as shown in Fig. 1. On the upper section B. coli from Case 17 urine is seeded. On the lower section B. coli from Case 18 urine is seeded, each with a nine-hour broth suspension of the respective strain. After drying in the incubator for three-quarters of an hour one or two drops of Case 17 bacteriophage filtrate is spread over the surface of the two areas avoiding contamination of one area with the other. The plate is now returned to the incubator for nine hours, or for overnight. At the end of this time it is examined for bacteriophage plaques. The upper section shows both large and small plaques—around the large plaque there is marked haziness while the plaque itself remains clear. Lysis is going on actively about its periphery. The small plaque is clean cut and clear without any haziness or lysis extending beyond its periphery. The lower section, which has been inoculated with the same bacteriophage filtrate, shows no large plaques while the small plaques are present in large numbers. They show no haziness. From this finding we are led to believe that the small plaque is the bacteriophage that is specific for this patient's (Case 18) B. coli. The left-hand section of the Petri dish is seeded with a third patient's strain and Case 17 bacteriophage filtrate is inocculated onto it. This serves as a control. No lysis occurs.

It will be seen that in developing the filtrate against Case 17 B. coli both types of plaques can be demonstrated, but against the patient's (Case 18) B. coli only the small plaque is active. Against control B. coli neither plaque is active. By carefully feeding the filtrate we finally enhanced the activity of the small plaque so that it became very numerous and very potent for Case 17 B. coli, and the large plaque very potent for Case 18.

Another observation of interest that shows the specificity of the bacteriophage was secured by taking a loop of nine-hour broth suspension that was lysed by the large plaque and not by the small plaque, and thoroughly mixing it with a loop of nine-hour broth suspension that lysed by the small plaque and not by the large, and seeding the mixture on agar in the usual manner. The bacteriophage filtrate (composed of both plaques) was then spread over the seeding. After nine hours' incubation the plate was studded with large and small plaques each acting on its specific organism.

CASE 18

DATE	CELLS	B. COLI	REMARKS
1931			
12-17	+	++++	Urine alkaline. Alkalinization begun 12-16-31
12-18			Slight costovertebral tenderness
12-19	±	++++	Urine alkaline. 3 c.c. filtrate in arm, 14 c.c. in bladder
12-21	0	±	Urine alkaline. 3 c.c. filtrate in arm, 14 c.c. in bladder
12-22			Clinical symptoms improving
12-23	0	0	Urine alkaline. 3 c.c. filtrate in arm, 14 c.c. in bladder
12-25	0	+	Urine alkaline
1932			
1-1	±	+	Urine alkaline. 2 c.c. filtrate in arm, 8 c.c. in bladder
1-3	0	±	Urine alkaline. 2 c.c. filtrate in arm, 8 c.c. in bladder. No growth on agar
1-27	0	+++R	Urine alkaline. 3 c.c. in arm, 8 c.c. in bladder. *Very Potent Phage.*
1-29	0	0	Urine alkaline.
3-19	0	0	Urine alkaline. 3 c.c. filtrate in arm. 8 c.c. in bladder.
3-21	0	0	Urine alkaline. 3 c.c. filtrate in arm. 8 c.c. in bladder.

ACUTE RECURRING COLON BACILLUS INFECTIONS

In this group (Table II) it will be observed that the bacteriophage acts in the same way as it does in the acute cases that occurred for the first time so far as we were able to judge. The sterilization of the urine was very prompt with the exception of the fourth case, which may have required nine days for sterilization. In a subsequent attack in this patient occurring seven months later, sterilization occurred promptly as it did in the other cases. It required only two days. The bacteriophage used at this time was very much more potent than it was in the first attack. In the column marked "Return of bacilluria" we, of course, mean that so far as we know there has been no return. Most of these patients would have reported to us had their been a recurrence. Case 4 may be regarded as cured of the attacks. If we look upon the bacteriophage simply as a means of sterilization, it has accomplished its mission when the urine becomes bacteria free, particularly if it remains free for any considerable number of days.

CASE 19.—Mrs. J. S., aged forty-two. C. H. December 31, 1924. Three days ago the patient complained of burning knifelike pain on micturition. This continued unabated. She observed blood in the urine on the morning of December 31. There was no fever or chills. There was a history of two previous attacks, the first several years before, and the second two years before. Urotropin treatment cleared up these attacks. They were preceded by an upper

TABLE II

ACUTE RECURRING INFECTIONS

NO.	AGE	SEX	APPROXIMATE DURATION BEFORE TREATMENT WITH BACTERIOPHAGE	URINE BECAME STERILE AFTER BEGINNING TREATMENT IN	NUMBER OF TREATMENTS	URINE REMAINED STERILE FOR AT LEAST	RETURN OF BACILLURIA AFTER	METHOD OF ADMINISTRATION	DATE OF ENTRANCE
19	42	F	3 days	3 days	3	4 days	No return	Subcutaneous	12-31-24
20	63	F	4+ weeks	3 days	3	26 days	No return	Subcutaneous	2-19-25
21	9	F		2 days	2	14 days	No return	Subcut. B	3-17-30
22	62	F	1 day	9 days	3		7 months	Subcutaneous	12-5-25
22	62	F	1 day	2 days	2	11 days	No return	Subcutaneous	6-19-25

respiratory infection. No known infection preceded the last attack. The urine on December 31 showed ++++ cells, some blood and B. coli. Alkalinization was begun. The patient responded promptly to treatment. The urine became sterile in three days. There has been no recurrence to date (1932).

CASE 19

DATE	CELLS	B. COLI	COLONIES	URINE	REMARKS
1925		R	S		
1-18	++++	+	+	acid	R = large regular homogeneous white colonies S = small bluish gray translucent colonies
1-20		+	+	acid	
1-21	++++				3 c.c. filtrate in arm
1-22	+	+	+	alk.	3 c.c. filtrate in arm. Symptoms have disappeared
1-23	+	+	+	alk.	3 c.c. filtrate in arm
1-24	+	0	0	alk.	
1-28	0	0	0	alk.	No return of symptoms

CASE 20.—Mrs. J. W. M., aged sixty-three. C. H. On February 19, 1925 the patient complained of inability to hold her urine and burning sensation on urination. These symptoms began four or five weeks previously. She was up frequently at night, and had to void about every two hours during the day. There had been no known recent infections. An ordinary urine specimen showed no pus and no albumin. Santol oil and increased fluids were ordered. The symptoms not improving she was given urotropin for a time and then alkalinization treatment.

On April 30, 1925 the patient reported that while here symptoms might be a little better she still occasionally had attacks of urinary frequency and burning. A catheterized specimen showed pus and B. coli. On May 1 alkalinization was begun. On May 9 the bacteriophage filtrate was satisfactory. Three doses of 3 c.c. each were given on alternate days. The result was marked. The symptoms promptly stopped, and the urine became sterile in three days. On June 7 the urine was negative. It was impossible to make further checks as the patient left for Europe. A careful check was run for us in Geneva on October 11, 1925. The urine sediment was reported negative, and the patient symptom-free. She contracted acute dysentery in Egypt February 2, 1927, and died February 9. The laboratory record follows.

CASE 20

DATE	CELLS	B. COLI	REMARKS
1925			
5-1	++++	+	Alkalinization begun April 30
5-9	++++		3 c.c. bacteriophage filtrate in arm. Arm swelled, painful
5-11	++	+	3 c.c. bacteriophage filtrate in arm. Reaction less marked
5-12		0	
5-13	++		3 c.c. bacteriophage filtrate in arm
5-15	+++		Cells disintegrating, mostly granular
5-29	++	0	Symptoms have all disappeared. Cells still increased
6-7		0	

CASE 21.—M. B., aged nine. U. H. 236520. March 17, 1930. Sent to Hospital without a history. Patient complained of costovertebral pain and tenderness. Had several attacks before. Physical examination except for pain on pressure in the left abdomen and reddening of the urethral orifice was negative. Temperature 103.8°. Respiration 25. Leucocytes 7,500. Duration of the condition could not be determined. The urine was loaded with pus and bacilli. Urine culture showed ++++ B. coli.

A satisfactory bacteriophage filtrate was developed in eight days. Recovery was prompt. Sterile urine was found on seven consecutive cultures. There has been no recurrence.

CASE 21

DATE	CELLS	B. COLI	CLINICAL RECORD
1930			
3-18	++++	++++	Alkalinization begun 3-17
3-20	++++		
3-25	++++	++++	
3-27	++++	++++	5 c.c. filtrate subcutaneously, 5 c.c. in bladder
3-28	0	+	
3-29	0	0	
3-31			5 c.c. filtrate subcutaneously, 5 c.c. in bladder
4-1	0	0	
4-2	0	0	
4-3	0	0	
4-5	0	0	
4-6	0	0	
4-10	0	0	Discharged on alkaline treatment

CASE 22.—Mrs. H. M. C., aged sixty-two. C. H. On November 11, 1925 the patient was taken ill with pain in the left hip. This continued for two weeks, and was diagnosed by her home physician sciatica. She occasionally had aching in this region. There were no other conditions that might be related to the complaints at this time.

December 5, 1925. On the morning of December 4 she awakened with "general distress." The bladder was distended. She passed an unusually large amount of urine. This was followed by a stinging sensation in the urethra. In 1919 she had had an attack of pyuria with B. coli positive, diagnosed pyelitis. At this time she had similar symptoms to those

CASE 22

DATE	CELLS	B. COLI	REMARKS
1925			
12-5	++++	++	Alkalinization begun
12-11			3 c.c. bacteriophage filtrate in arm
12-12			
12-13			3 c.c. filtrate in arm
12-14			
12-15			3 c.c. filtrate in arm
12-20	0	0	Symptoms disappeared after second injection

previously described. A catheterized urine specimen on December 5 showed several hundred leucocytes per low power uncentrifuged field, many large clumps. Culture showed B. coli. Alkalinization was begun.

The patient continued to be free from symptoms until June 19, 1926. When there was a recurrence of urinary irritation she was again alkalinized and given a second course of bacteriophage injections. The urine sterilization was quite prompt as shown in the record. This patient has had three distinct attacks. The reinfection after previous bacteriophage treatment was promptly overcome by a second series of injections. This illustrates that one course of bacteriophage treatment does not build up effective phagocidal antibodies.

CASE 22

DATE	B. COLI R	B. COLI S	URINE P. H.	REMARKS
1926				
6-20	+	+	6.5 acid	Alkalinization begun
6-22	+	+	7.5 alk.	
6-23				3 c.c. filtrate in arm
6-24		+	7.6 alk.	
6-25	0	0	8.2 alk.	2 c.c. filtrate in arm
6-26	0	0	7.4 alk.	
6-29	0	0	7.5 alk.	
7-2	0	0	7.4 alk.	
7-6	0	0	7.4 alk.	

CHRONIC COLON BACILLUS INFECTIONS

In order to form a proper idea of the effect of the bacteriophage in the chronic cases recorded, each case should be studied by itself. It is possible to group 11 cases together as has been done in Table III in which recovery occur-

TABLE III

CHRONIC COLON BACILLUS INFECTIONS RECOVERY WITHOUT KNOWN RECURRENCE

NO.	AGE	SEX	APPROXIMATE DURATION BEFORE TREATMENT WITH BACTERIOPHAGE	URINE BECAME STERILE AFTER BEGINNING TREATMENT IN	NUMBER OF TREATMENTS	LAST OPPORTUNITY TO TEST AND FIND URINE STERILE	LAST REPORTED RECORDING NO RECURRENCE OF SYMPTOMS	DATE OF ENTRANCE
27	5	F	6 months	2 days	3	4 days	1 month	10-11-26
29	8	F	3 years	1 day	3	6 days	No return	7-8-28
31		F	Many months	6 days	6	12 days	Several months	9-18-28
32	60	F	Many months	513 days	30	320 days	Two years	11-27-28
37	18 m.	F	2 months	50 days	6	3 days	No return	7-29-29
39	7	F	4 months	4 days	4	15 days	No return	10-28-29
40	35	F	Many months	5 days	6	4 months	No return	10-29-29
42	2	F	Several months	47 days	3	39 days	No return	8-6-30
45	50	F	Many months	32 days	4	7 days	5 months	5-25-31
35	50	M	30 years	455 days	46	1 month	12 months	6-6-29
34	20	F	Months	1 day*	4	79 days	Two years	1-17-30

*This patient had previously had two courses of bacteriophage treatment without any beneficial effect. Four months elapsed between the second and third courses.

red without any known recurrence after discharge. The records of two of these, Cases 10 and 12, are of particular interest. One is of over two years' duration, the other of probably thirty years. They illustrate very well what persistence in treatment and in cooperation from the patient will accomplish. They seem to preclude the idea of the long result of time, of alkalinization or of both being responsible for the entirely satisfactory result.

There are 6 cases of infections recurring after the first series of treatments, Cases 23, 26, 28, 30, 32, and 44, which finally recovered under the influence of the bacteriophage. These recurrences occurred as early as six days and as late as three and a half months, after the first or second series of injections. There are four cases, Cases 25, 38, 43, and 46, which recovered symptomatically, and which, as long as we were able to follow them bacteriologically, continued to show a bacilluria. In all of these there was a modification of the bacilluria or a decrease in the number of bacteria which may be attributable to the bacteriophage. There are three cases (Cases 24, 36, and 41) in which there was no improvement in the bacilluria or in the clinical condition following the administration of bacteriophage. An abridged detailed record of these cases follows.

CASE 23.—Mrs. J. O., aged fifty-two. C. H. October 6, 1923. The patient complained of a "feeling of pressure" and an occasional sharp piercing pain in the "neck of the bladder." There was no burning or unusual sensation on micturition. These symptoms occurred off and on for a long time. An uncentrifuged urine specimen showed 100 cells per low power field. There had been no previous infections. She had had similar symptoms before. At one time they were associated with fever and what was diagnosed erythema nodosum.

The physical examination was negative as far as its relation to urinary tract infection was concerned. There was a moderate rectocele which was giving no trouble. The meatus urinerius was smooth and not injected. On October 12 a catheterized specimen showed B. coli, and an excess of leucocytes. X-ray examination was suggestive of gall bladder disease.

Catheterized urine specimens on October 12, November 19, December 12, 1923, and on January 5, 1924 showed an excess of leucocytes varying from a definite excess with clumps to five or six cells per low power field on January 5. The patient was treated with santol oil for a time, with urotropin and with alkalinization. On March 3, May 22 and June 2 urine samples were free from leucocytes in excess and the patient had been free from symptoms for a long time.

On June 19, 1925 the patient again became conscious of some urinary disturbance. A catheterized specimen showed definite pus and many motile bacilli. Culture showed B. coli.

CASE 23

DATE	CELLS	B. COLI	URINE P.H.	REMARKS
1925				
6-19	++++	+	acid	Resistant type, large convex mucoid colonies
6-23				3 c.c. bacteriophage filtrate in arm
6-24		+	8.0 alk.	Same type of colony
6-25		+	8.0 alk.	2 c.c. bacteriophage filtrate in arm
6-26		+	8.2 alk.	Some flat irregular colonies
6-28				2 c.c. bacteriophage filtrate in arm
6-29		+	7.8 alk.	Colonies all large, very irregular, moth eaten
7-3		+	8.8 alk.	Only large flat irregular moth eaten type of colonies remain
7-6		+	7.6 alk.	Colonies same as 7-3
7-9		0	7.6 alk.	11 days after last treatment
7-14	0	0	7.8 alk.	16 days after last treatment

Bacteriophage treatment was begun June 23, 1925. Records of the cell content of the urine unfortunately were not kept. Interest was focused on the bacteriology of the urine. The patient's symptoms cleared up and she was much better generally. It will be observed that eleven days after the last injection of the bacteriophage filtrate the urine became sterile and pus had disappeared at least by the sixteenth day. The result of this series of treatments seemed highly successful. There was no recurrence of symptoms for six months.

On January 15, 1926 a recurrence of symptoms after six months occurred. The progress is recorded in the following table.

CASE 23

DATE	CELLS	B. COLI	REMARKS
1926			
1-15	++	+	Alkalinization begun
1-17	±		3 c.c. bacteriophage filtrate in arm
1-19			3 c.c. bacteriophage filtrate in arm
1-21	++		
1-23	±		Symptoms improved
3-17	+	+	Mild symptoms
3-21	+		3 c.c. bacteriophage filtrate in arm
3-22	+	+	Catheterized specimen
3-23			3 c.c. bacteriophage filtrate in arm
3-24		+	Catheterized specimen
3-25			3 c.c. bacteriophage filtrate in arm
3-26		+	Catheterized specimen
4-2			Catheterized specimen. Record lost
5-5	0	±	Symptoms have disappeared
5-13			3 c.c. bacteriophage filtrate in arm
5-14		±	Catheterized specimen
5-15			3 c.c. bacteriophage filtrate in arm
5-17			3 c.c. bacteriophage filtrate in arm
5-18	0		Catheterized specimen. Symptoms have disappeared
7-9			3 c.c. bacteriophage filtrate in arm
7-10		±	
7-11			3 c.c. bacteriophage filtrate in arm
7-12	0	±	Catheterized specimen shows a modified atypical organism
7-13			3 c.c. bacteriophage filtrate. Symptom free
10-7	++	+	Alkalinization continued. Recurrence of symptoms after 3 months.
10-12			3 c.c. bacteriophage filtrate in arm
10-14		++	
10-15			3 c.c. bacteriophage filtrate in arm
10-16		+	
10-17			3 c.c. bacteriophage filtrate in arm
10-18		+	
11-5	0	+	Symptoms have disappeared
12-4		0	
1927			
3-22	0	0	Catheterized specimen
3-23	0		Ordinary specimen. Symptoms have not returned

Comments.—This case illustrates very well the persistence of B. coli infections. It also illustrates very well the fact that bacteriophage treatment does not impart immunity, and that a negative culture can be brought about after several recurrences of colon bacilluria. It cannot be said that the urinary tract was ever completely sterilized or whether there had been several reinfections of the urinary tract from the original source. The fact that definite symptoms and definite excess of cells recurred, as well as bacilluria, seems to indicate that there was a definite resistant continuous infection present. The presence of colon bacilli was too constant to enable us to attribute their presence to the simple elimination or filtration of organisms which might normally enter the blood stream or the lymphatics and through these channels gain entrance to the urinary passages.

At the time of the first recurrence of symptoms, January, 1926, we had on hand an unusually vigorous polyvalent bacteriophage. We were, however, unable to increase its potency for the patient's strain. A new river water sewage sample was taken. In time we were able to develop a bacteriophage that showed many areas when tested against the patient's strain on solid media in dilutions as high as 1: 10,000,000. Both alpha and beta plaques were present.

It will be observed that a prolonged effort over a period of ten months was unsuccessful in causing the organism to disappear from the urine. Its numbers were at times reduced and the patient's symptoms disappeared. After this prolonged effort one would be glad to accept the last two negative cultures (December 4, 1926 and March 22, 1927) as evidence of the efficiency of the bacteriophage treatment. This may be questioned. However, the patient remained free from symptoms for a long time.

The events in this case may lend support to the opinion that the first course of treatment which apparently resulted successfully rendered further use of the bacteriophage useless, because of the development of antibacteriophage antibodies. If the body had developed antibodies against the activity of the bacteriophage, it may be that these antibodies would be found in the patient's blood serum. We attempted to prove whether this was so. We ran a series of dilutions of the bacteriophage with the patient's serum. Even in the highest dilutions where only a trace of bacteriophage was present, the serum proved to be innocuous. In no instance did the bacteriophage show any loss of potency over that diluted in a similar way with broth or with normal human serum. Bordet and Ciuca* mixed rabbit serum and coliphage together and seeded the mixture with B. coli. The colon bacillus grew as luxuriantly as it did in plain broth. This is not analogous to the inoculation of a case of B. coli infection with bacteriophage.

An attempt was made on several occasions to demonstrate bacteriophage in the patient's urine. Only at one time, following the first injection (1926) were we able to do so. In this instance large numbers of plaques were found. Their potency we thought was even higher than it was before injection.

Case 24.—Katherine H., aged three. U. H. 242609. November 5, 1925. This was a marked case of nephrosis with a colon infection of the urinary tract of long standing which had resisted alkaline and forced fluid treatment. The catheterized urine specimens on November 5, 8, 12, 13, 15, and 17 contained pus and large numbers of B. coli. After three subcutaneous injections of 3 c.c. of bacteriophage filtrate which imperfectly lysed the patient's organism no beneficial effect was secured. Later on a second series of three injections was given. The urine culture became negative and remained so for sixteen days, after which B. coli again appeared. We were never able to clear up this infection. Subsequently the

Case 25

DATE	CELLS	B. COLI	REMARKS
1926			
1-26	++++		Santol oil 5 min. t.i.d. Forced fluids
2-5	0		Catheterized specimen. Negative. No symptoms
4-5			Sudden uncontrollable desire to urinate
1927			
2-21	0	+	
3-2	0	+	Alkalinization begun
3-15			3 c.c. bacteriophage filtrate in arm
3-17	0	+	3 c.c. bacteriophage filtrate in arm
3-19	0	+	3 c.c. bacteriophage filtrate in arm
3-24	0	±	
3-31	0		Symptoms have disappeared
4-14	0		
4-16	0	+	
4-18	0	+	
4-20	0	+	
5-2	0	+	
5-25	0	+	

*Cited by D'Herelle.

patient died. At autopsy there was no microscopic evidence of involvement of the kidney pelvis.

CASE 25.—Mrs. S., aged thirty. C. H. January 27, 1926. The patient came in with a history of painful micturition and frequency of two weeks' duration, preceded by an acute upper respiratory infection. Physical examination was negative aside from the discovery of a small smooth firm pelvic mass the size of a large walnut, separated freely from the uterus and ovary. There was a history of a dermoid cyst having been removed two years previously. A catheterized urine specimen showed 100 to 120 cells per low power field, no casts, no blood, chemistry normal, B. coli ++++, R. type, indicating an old infection.

Final Report: The symptoms quite promptly disappeared. They reappeared October, 1929. We were unable to sterilize this patient's genitourinary tract with three injections of bacteriophage. Cystoscopic examination and pyelograms showed no malformations. The organism encountered was the R. type of B. coli. At no time did we discover a bacteriophage in this patient's urine.

CASE 26.—Mrs. G. W., aged forty-eight. C. H. July 18, 1926. The patient had been having nausea, vomiting, fever, and chilliness for a week or ten days, and showed slight but definite jaundice. There was no characteristic tenderness in the gall bladder region. The symptoms were suggestive of acute gall bladder disease. There were no urinary symptoms. The patient was very ill. By July 28 the patient was much better but still vomited every day, particularly after the noon meal. Putting her on thick, smooth food at this time improved this symptom. On August 11 the husband wrote that she had made rapid gains. The nausea and vomiting had ceased entirely. She had been sitting up a little each day, but had developed a burning sensation on micturition. On August 14 an attack of vomiting and fever of 103.6° occurred. This cleared up in two days.

September 7, 1926. The patient entered the Hospital. She was too ill to undergo a complete examination. She had lost a great deal of weight, was weak and emotional, cried on the slightest provocation. The general bed examination showed nothing else. No tumor mass could be felt in the abdomen. There was no tenderness in the gall bladder region and pelvic examination was negative. A urine sample showed no increased cells; the chemistry was negative. Later in the day a catheterized specimen was taken for culture. It contained pus. This was of interest in view of the fact that pus had not been found before. Careful check showed that the first sample had not been confused with that of another patient. She was put on

CASE 26

DATE	CELLS	B. COLI	REMARKS
1926			
9-7	++++	+++	Santol oil 5 minims after meals
9-9			Bladder irrigated with boric acid sol., 20 per cent argyrol instillation
9-13			Alkalinization begun
9-17			Bladder irrigated with boric acid, 20 per cent argyrol instillation
9-19			Bacteriophage only partially lytic for case strain B. coli
9-20			Bladder irrigated and argyrol instillation
9-27			Bladder irrigated and argyrol instillation
9-28			Bladder irrigated and argyrol instillation
10-4	++++	+++	Patient continues to remain in bed
10-16	++++		3 c.c. bacteriophage filtrate in arm
10-17	++++		3 c.c. bacteriophage filtrate in arm
10-18	++++		3 c.c. bacteriophage filtrate in arm
10-26	++	+	B. coli colonies greatly reduced in number
11-1	++	+	Patient up and about. Walks down town easily. Gaining in strength
11-16	++	+	Has gained 14 pounds in weight. Returned home on hexylresorcinol treatment
1927			
1-12	0	+	
2-22		0	Four months after last bacteriophage injection
3-10		0	Patient is very well and active. Has gained 18 pounds in weight. No recurrence of symptoms

Santol oil pending the development of bacteriophage. We were never able to build a bacteriophage that would completely lyse this patient's organism in the test tube. There was a decrease in the number of organisms following the bacteriophage injections, and the patient made very rapid gains after this time, which we think may be attributed to the bacteriophage. It should be noted, however, that the urine did not become sterile until four months after beginning treatment. This may be attributed to hexylresoreinal. There was a marked effect on the bacterial count ten days after beginning bacteriophage treatment.

Follow-Up Record.—May 28, 1928. Patient returns on request for further observations. She continues to be well and has no urinary complaints. A catheterized urine specimen, however, showed pus and B. coli of two types, large and tiny colonies. One of our stock bacteriophage filtrates was potent for the patient's organism. Alkalinization was begun. Because of this long continued infection we were interested to know if a bacteriophage was living symbiotically with this patient's B. coli. We were unable to demonstrate one before beginning the filtrate injections. The following observations were made.

CASE 26

DATE	CELLS	B. COLI LARGE	COLONIES TINY	REMARKS
1928		S	R	
5-28	+	++++	++++	Bacteriophage adaption begun
6-22	+	++++	++++	3 c.c. filtrate in arm. No bacteriophage found in urine
	+	+++	++++	One hour after treatment. No bacteriophage found in urine
6-24		0	++++	3 c.c. filtrate in arm. No bacteriophage found in urine
		0	++	One hour after treatment. No bacteriophage found in urine
6-26		0	++	3 c.c. filtrate in arm.
	0	0	++	One hour after treatment. No bacteriophage found in urine
6-29		0	+	3 c.c. filtrate in arm. No phage in urine after 24 hours. Abundant after 48 hours
		0	+	One hour after last treatment. No phage in this sample of urine after 24 hours. Abundant plaques after 30 hours.

These observations are of interest in that the sensitive strain or type of B. coli in this patient's urinary tract was easily caused to disappear. The tiny or resistant type persisted although its numbers were definitely reduced. They also show that the bacteriophage after being injected subcutaneously may later appear in the urine. In this case it required thirty to forty-eight hours before it could be demonstrated. The bacteriophage filtrate used in this patient was potent for several case strains of B. coli in dilutions up to one to ten thousand.

CASE 27.—Dorothy D., aged five. U. H. 155247. October 11, 1926. The patient was admitted to the Hospital because of albuminuria, casts, and pus in the urine. In April, 1925 she developed a fever without any other symptoms, which continued for two months. There was considerable loss of weight. The fourth week of this illness casts and albumin in the urine were discovered. These findings persisted. There was no urinary frequency, no hematuria, no edema, nocturia or incontinence. Recurring attacks of dull pain in the left upper abdomen have occurred since June, 1925. These sometimes last for twenty-four hours. No vomiting or fever. Last attack occurred two weeks previous to entrance.

Physical examination: Evidence of loss of weight; otherwise negative. Blood: hemoglobin 75 per cent, red cells 3,400,000, white cells 8,100. X-ray of chest negative. Upper respiratory infection present. "Cold."

November 23, 1926. Mother reported a good weight increase, better color, exceptional appetite. Prior to treatment appetite was very poor.

CASE 27

DATE	CELLS	B. COLI	REMARKS
1926			
10-11	+++	+++	
10-22			Alkalinization begun.
10-23	++		3 c.c. filtrate in arm. Marked reaction. Temp. 104° swelling, redness, pain in leg
10-25	±	0	2 c.c. filtrate in arm. Reaction less marked
10-27	0	0	3 c.c. filtrate in arm. Urine alkaline
10-29	0	0	

CASE 28.—Mrs. W. H. A., aged fifty. C. H. October 17, 1927. Several days ago the patient began to complain of painful micturition. A catheterized urine specimen showed many leucocytes, no casts, and on culture ++++ B. coli. Otherwise this patient is in excellent health. Alkalinization was begun. It is possible that she previously had a similar infection.

CASE 28

DATE	CELLS	B. COLI		REMARKS
1927				
10-17	++++	++++	S	Sensitive or lytic type of colony. Alkalinization
10-18	++++			
10-19	++++			
10-20	++++			
10-21				3 c.c. filtrate in arm (diluted filtrate)
10-22		0		
10-23	++	0		3 c.c. filtrate in arm
10-25	+++			3 c.c. filtrate in arm
10-27	++++	0		
11-1	++++	+	R	Resistant type of colony has developed
11-3	+++	±		3 c.c. filtrate in arm (undiluted filtrate)
11-5	++	±		3 c.c. filtrate in arm (produced marked local reaction)
11-7				3 c.c. filtrate in arm
11-9	+++			
11-14	++			Argyrol instillation into bladder (25%)
11-29	0	0		Symptoms have disappeared

The change in the character of this patient's urine after the first dose of bacteriophage was remarkable. The symptoms were also improved. There was a greater improvement in the bacteriology than in the cytology. The urine remained sterile for nine or ten days when it again became positive for B. coli. An entirely different organism than the original one was now present, a typical rough bordered colony, the so-called R. type, the organisms of which were self-agglutinating. On November 29, thirty-nine days after the first dose of bacteriophage, thirty-four days after the last, a catheterized urine specimen was negative bacteriologically and cytologically. The patient continued to be well. However, the clearing up of the urine was preceded fifteen days by an instillation of argyrol, at which time a culture test was not run. In February, 1928, after a symptomless period of four months, there was a recurrence of the former symptoms.

CASE 28

DATE	CELLS	B. COLI	REMARKS
1928			
2-28	++++	+	Alkalinization begun
3-11			3 c.c. bacteriophage filtrate in arm
3-13			3 c.c. bacteriophage filtrate in arm
3-14	0	0	
3-16			3 c.c. bacteriophage filtrate in arm
3-17	0	0	Arm sore, swollen, chilliness, aching

The response to bacteriophage again was rapid. It is instructive to follow the course of this case still further. On September 17, 1928 the patient complained of urethral irritation. Examination revealed a slightly irritated urethral caruncle, which responded to cauterization with silver nitrate. In November irritation again developed with excess of leucocytes and red blood cells in the urine. A catheterized specimen was negative for cells. They were coming from the meatus. This situation improved on a second cauterization. On November 21 irritation and increased cells occurred again. A catheterized specimen was negative for cells. The caruncle was removed by electrocoagulation. On December 7, 15, January 9 and 29, 1929, urine samples remained cytologically negative. On April 30, and May 27 specimens were negative. On June 3 and 22 ++ cells were present, July 29 negative, December 3 negative, January 7, 1930 cells +++, B. coli negative, January 11 B. coli negative. On April 25, 1931 small polypoid growths in the urethra, slightly irritated, were found, but none of the symptoms associated with the former bacilluria were present.

This case is cited at length to show that it is wise to differentiate the causes of urinary symptoms and findings in cases that have been treated with bacteriophage. One might easily in this case have made up his mind that after all the bacteriophage accomplished little—if he trusted the ordinary urinary findings and the symptom of urethral irritation. Inspection and catheterization are necessary.

CASE 29.—Irene R., aged eight. U. H. 197384. July 8, 1928. Patient brought to Hospital for tonsil and adenoid removal. Three years ago appetite became poor. On several occasions she vomited after heavy meals. Riding on street cars nauseated her. Aside from septic tonsils and evidence of mental retardation the examination was negative. Kahn negative. Bed wetting began about three years ago.

September 12, 1928. Night sweats for past two weeks, at which time she began to have chills, followed by lassitude and loss of weight. Nausea followed the chills. Complained of pain in right chest when she breathed, afternoon fever, and drenching sweats. Examination revealed tenderness in the right lower quadrant of the abdomen with muscle spasm, slight general adenopathy, decreased breath sounds, right axillary line near nipple level. Tuberculin negative. X-ray of lungs showed an increase in the hilum shadows not typical of tuberculosis. Blood pressure 110/90.

Blood chemistry: chlorides 474, serum protein 7.6, albumin 4.8, globulin 2.8, cholesterol 0.208, phenolsulphonephthalein negative, nonprotein nitrogen normal.

October 7, 1928. Urine showed cells ++++, B. coli ++++. Three stains for tubercle bacilli negative. Pig inoculated (result was negative). B. coli positive. Condensed record follows.

CASE 29

DATE	CELLS	B. COLI	CLINICAL NOTES
1928			
10-7	++++	++++	Alkalinization begun
10-8	++++	++++	Phage development begun
10-10			Temp. 102°. Restless, eyes puffy
10-11	++++		
10-13	++++	++++	
10-20	++++	++++	
10-24	++++	++++	
10-31	++++	++++	
11-8	++++	++++	
11-10			Urotropin and sodium acid phos. Alkali begun again Nov. 17, 1928
12-11	++++	++++	3 c.c. filtrate in arm, 3 c.c. in bladder. Very active phage
12-12	0	+	20 hours after treatment
12-13			3 c.c. filtrate in arm, 5 c.c. in bladder
12-14	0	0	No phage could be recovered from urine
12-15			3 c.c. filtrate in arm, 5 c.c. in bladder
12-16	0	0	
12-17	0	0	
12-18	0	0	
12-21	0	0	

Bacteriology.—Three times this patient went through the ordinary process of alkalinization preparatory to bacteriophage treatment and for some unexplainable reason the filtrate was not given. This alkalinization had no effect in lessening the number of B. coli or in changing their colony characteristics. Several methods of treatment were tried without benefit. The bacteriophage filtrate was potent six weeks before it was used for treatment. During this time its titer of potency was constantly raised by successive feedings. It became highly polyvalent and was used as a base for other cases. Its action after subcutaneous injection and instillation was very energetic. It caused the pus to disappear from the urine in twenty hours after the first injection, and the number of organisms to change from ++++ to +. Twenty-four hours after the second injection the urine was sterile and remained so.

Case 30.—Andrew W., aged eleven. U. H. 198774. July 24, 1928. Patient came to the Hospital because of a sore mouth. On July 21 the patient had a chill, and on the following day had chills, fever, delirium, and malaise. These symptoms continued for three days. He complained of his teeth, mouth, and tongue being sore. The mother described sores on the tongue and buccal mucous membranes. He also told that one week previous a cousin with whom the patient had been playing had the same mouth infection.

On entrance small white patches were found on the upper left tonsil, an ulcerated spot on the buccal mucous membrane, and several small ulcerated patches on the gum margins. There was a marked left cervical adenitis. Aside from this and the general appearance of illness the examination was negative. X-ray of the chest was negative. Temperature 102°.

August 9, 1928. Smears from the ulcerated surface were negative. Repeated urine examinations were negative. Patient discharged. Diagnosis: ulcerative stomatitis.

August 29, 1928. Readmitted. There had been an attack of fever, nausea and delirium in November lasting four days. Two similar attacks had occurred since that time. Patient was fairly well between these attacks. During the last six weeks there had been puffiness of the eyes. There had been urinary frequency and burning at times. On August 28, 1928 there was right costovertebral angle pain, fever and delirium. Patient was poorly

Case 30

DATE	CELLS	B. COLI	URINE FINDINGS—CLINICAL NOTES
1928			
8-30	++++	++++	
9-16	++++	++++	Bacteriophage development begun
9-20	+		
9-23	±		
9-24		++++	
9-26	++++	++++	
10-8	++++	++++	Control for treatment not satisfactory. Moderately potent phage developed
10-9	++++	++++	3 c.c. filtrate subcu. Severe general and local reaction. Temp. 103+°
10-10	++++	++++	3 c.c. filtrate subcu. Severe general and local reaction
10-11	+	++++	
10-12			3 c.c. filtrate subcu. Severe general and local reaction.
10-13	±	+++	
10-14	0	±	
10-16	0	+	
10-21	++	+++	
10-26	±	++	3 c.c. filtrate subcu. Severe general and local reaction
10-28		±	
10-30	0		
11-3	+++	++++	
11-4	+++	++++	
11-5	+++	++++	
11-6	+++	++++	
11-7			Cystoscopic. Left pyelogram. No pelvis retention.
11-12			Right pyelogram. Slight dilatation of ureter. Patient has had alkalinization treatment all this time.

CASE 30 (Cont'd)

DATE	CELLS	B. COLI	URINE FINDINGS—CLINICAL NOTES
1929			
8-29			Discharged on sodium bicarbonate for fourteen days with four-day intervals without carbonate
1930			
3-10			Readmitted. Repeated febrile attacks since discharge
3-12			Tonsillectomy and adenoidectomy
3-15			Discharged from Otology Service
3-30			Readmitted. High fever past five days. Urine pus. No casts. No alb.
4-10	+	++++	*New phage. Very potent* for present clinical strain. Filtrate has been under constant development since last treatment. Alkalis have been given continuously
4-29	++	++++	3 c.c. filtrate subcutaneously, 5 c.c. in bladder
4-30	+	±	Marked local and general reaction
5-1	±	±	Missed treatment
5-5	++	+++	3 c.c. filtrate in arm, 5 c.c. in bladder. Reaction marked
5-6	++	+++	
5-7	±	±	0 c.c. filtrate in arm, 5 c.c. in bladder. Reaction none
5-8	0	0	3 c.c. filtrate in arm, 5 c.c. in bladder. Reaction marked
5-9	0	0	
5-10	0	0	
5-11	0	0	
5-12	0	0	
5-16	0	0	
5-19	0	0	
5-21	0	0	
5-22			3 c.c. filtrate in arm, 5 c.c. in bladder. Reaction marked
5-23	0	0	
5-24	0	0	
5-25			Discharged on alkaline treatment
7-29	0		Returns for check. Urine not sent for culture. Returned home
1931			
2-11	0		Returns. Several attacks of gradually increasing nervous manifestations. Very irritable. Pain in right leg and costovertebral angle for 2-3 weeks. Urine not sent for culture

nourished, looked chronically ill. There was puffiness of the eyes and tenderness in the right flank. Tonsils were septic. Kahn negative.

August 30, 1928. Urine showed pus and B. coli ++++.

This case illustrates the necessity of securing an unquestionably potent bacteriophage for treatment. From August 30, 1928 to April 29, 1930, a period of twenty-two months, no change in the number or virulence of B. coli was observed after twenty cultures, excepting following the first series of bacteriophage treatments with a filtrate we recognized as unsatisfactory. Two days after the third injection the culture changed from ++++ to ± and the cells from ++++ to 0. The original picture, however, was again established in two weeks.

CASE 31

DATE	CELLS	B. COLI	REMARKS
1929			
9-18	++	++++	3 c.c. filtrate in arm
9-19	++++	++++	
9-20	+++	++++	3 c.c. filtrate in arm
9-22	+++	++	3 c.c. filtrate in arm
9-24	0	0	3 c.c. filtrate in arm*
9-26	0	0	3 c.c. filtrate in arm
9-28	0	0	3 c.c. filtrate in arm
10-6	0		Discharged

*Through a misunderstanding patient stopped soda when bacteriophage treatments were begun. Resumed.

The entirely satisfactory bacteriophage filtrate given April 29 and May 3, 1930, brought about prompt sterilization of the urine. The urine remained sterile on ten successive cultures over a period of sixteen days after which the patient was discharged.

CASE 31.—Mrs. B., aged ??. C. H. September 18, 1928. Sent to Hospital for bacteriophage treatment for pyuria and bacilluria, which has resisted other forms of treatment for many months. She has been taking soda for several days preparatory to treatment. The urine is alkaline. There is a slight excess of cells. She is now feeling better because of the soda. A satisfactory phage was found among our stock filtrates. Recovery was prompt without recurrence.

CASE 32.—Mrs. J. D., aged sixty. C. H. November 30, 1928. Patient has been under treatment for pyuria for many months. It is not possible to determine the exact duration of the infection. Urotropin, hexylresorcinal and infusion of buchu treatments have been employed. The pyuria has partially cleared up but the bacilluria continues. She is conscious at times of urinary irritation and occasionally of urinary frequency. There has been a bad odor to the urine for a long time.

This patient came under our observation because of bronchial asthma which was found to be on a multiple sensitization basis. Only the record of her urinary infection is recorded. Aside from the slight but definite incapacity of age incident on these two conditions the physical examination has no bearing on the urinary infection. The urine contained pus and many bacteria. The first catheterized specimen was taken November 26, 1928. It showed ++++ cells and ++++ B. coli. On Endos media the colonies were 2 mm. in diameter and were characteristic of B. coli. The organism reacted to dextrose, lactose, saccharose and on dulcite for B. coli communior characteristically.

Bacteriophage adaptation was very difficult in this case. On constant planting from November 27, 1928 until July, 1929 only a bacteriophage of weak potency was developed. This was given with the hope that it might be enhanced in the patient.* A perusal of the treatment record will show that nothing was accomplished in a period of ten months excepting in the cell content of the urine and statements from the patient that she was better. In fact, she was more encouraged than we were.

In September, 1929 we obtained the first bacteriophage that seemed satisfactory. It was very potent for the patient's organism. There was a prompt clearing of the patient's urine. The catheterized specimen showed no excess of cells two days after the first subcutaneous injection. After the third injection bacteria in the direct drop were negative, and the culture dropped from ++++ to +. From this time on the cell content was normal, but the organisms were slow in disappearing. The urine finally became sterile and the patient has remained symptom free until the last report on January 4, 1932.

The patient was treated for over one year before any remarkable change occurred in the bacilluria. Consequent on the securing of a potent bacteriophage marked improvement began. Alkalinization was kept up almost constantly. The detailed treatment record follows.

CASE 32

DATE	L.P.*		CULTURE	FILTRATE		
1928	CELLS	BACT.	B. COLI	S.Q.	BLAD.	REMARKS
11-27	++++	++++	++++			Marked urine odor. Alkalinization begun
12-5	++++	++++	++++	3 c.c.		Stock phage. No effect on patient's B. coli. Special filtrate building
12-7	++	++++	++++	3 c.c.		
12-9	++	++++	++++	3 c.c.		
12-12	+	++++	+++	3 c.c.		
1929						
1-3			++++			
1-8				3 c.c.		
1-12	++			3 c.c.		

*Low power.

*Cowie, D. M.: Observations on the Bacteriophage, Ann. Clin. Med. 1: 73, 1926.

CASE 32 (Cont'd)

DATE		L.P.*	CULTURE	FILTRATE		
1929	CELLS	BACT.	B. COLI	S.Q.	BLAD.	REMARKS
3-30	+	+++				
4-10	++++		++++			
4-28	++++		++++			
5-6	+	+++				
5-22	++++	++++	++++			More cells and bacteria than seen before
6-12	++++	++++	++++			
6-20			++++	3 c.c.		First injection second series. Bacteriophage still weak
6-22	++	0	++++	3 c.c.		
6-24	±		++++	3 c.c.		
7-10	++		++++	3 c.c.		Weak phage used third series
7-12	+++		++++	3 c.c.		
7-14	0	0	++	3 c.c.		
7-15	0	0	0			
7-24	++	+++	++++	3 c.c.		Weak phage fourth series
7-26	+++	+++	++++	3 c.c.		
7-29	+	+++	++++	3 c.c.		
9-5	++	+++	++++	3 c.c.		Fifth series filtrate very potent. First obtained potent for patient's organism. Constant development since Nov. 27, 1928
9-7	0	++	++++	3 c.c.		Urine odor has disappeared
9-9	0		++++	3 c.c.		
9-10	0	0	+			24 hours after third injection
9-11			±	3 c.c.		
10-8	0		++++	3 c.c.		6th series. Filtrate being enhanced by feeding every day
10-10	0		+++	3 c.c.		
10-12			+	3 c.c.		
10-14	0		+++	3 c.c.		
10-19	0		+++	3 c.c.		
10-25	+		+++	3 c.c.		
11-1	0		++++	3 c.c.		
11-8	0		*+++	3 c.c.		
11-15	0		+++	3 c.c.		
11-27	0		++++	3 c.c.		
12-6	0	0	+			
12-20	0	0		3 c.c.		
1930						
1-3	0	0	++	3 c.c.		
1-14	0	0	0	3 c.c.	10 c.c.	
1-21	0	0	0	3 c.c.	10 c.c.	
1-28	0		±	3 c.c.	10 c.c.	Two colonies B. coli
2-5	0		0			
4-23	0	0	+			
4-30	0	0	0	3 c.c.	10 c.c.	
5-7	0	0	±			An entirely different organism
5-14	0	0	0	3 c.c.	10 c.c.	
6-4	0	0	0			
12-4	0	0	0			No further symptoms. Recent urine sample shows no excess leucocytes

*Low power.

CASE 33.—Mary J. N., aged seven. C. H. April 26, 1929. The patient has complained of bed wetting and urinary frequency spells lasting for two weeks at a time with remissions for variable periods. This situation has been going on for many months. Pus in the urine has been reported. There has been no fever. The patient is extremely nervous and excitable during the bed wetting periods and has frequent nose bleeds. The remissions may be as long as six months, during which time her irritability disappears. The attacks begin with suddenly developing uncontrollable urination and urinary frequency. If at these times she endeavors to hold her urine it causes great discomfort. There seems to be no background for the urinary infection. She is a robust youngster and has had none of the

contagious diseases and no upper respiratory infections. A catheterized urine specimen showed a great excess of leucocytes and B. coli. Alkalinization begun.

CASE 33

DATE	CELLS	B. COLI	REMARKS
1929			
4-26	+++	++++	Alkalinization begun
5-17	0	++++	
5-28			3 c.c. filtrate in arm
5-29	0	++++	
5-30			3 c.c. filtrate in arm
5-31		++++	
6-1			3 c.c. filtrate in arm
6-2		++++	
6-10	+++		Catheterized urine specimen
6-13	±		Catheterized urine specimen
6-15		+	
6-21	++++	+	

We had great difficulty in treating this child. We had to discontinue the injections because of poor cooperation. The injections we gave were forced. There was noticeable improvement in the bacterial count. She was put on urotropin treatment for two weeks, then back on alkalinization. On September 9, 1929 the mother wrote that the child had had one of the severest attacks she had ever witnessed. Advised return once a week for bacteriophage treatment. The record follows.

CASE 33

DATE	CELLS	B. COLI	REMARKS
1929			
9-16	++++		Alkalinization continued
9-28	++++		
10-7	±	+	
10-12	0	+	
1930			
1-3			3 c.c. filtrate in arm, 10 c.c. in bladder
1-4		0	
1-11	+		3 c.c. filtrate in arm, 10 c.c. in bladder
1-18	±		3 c.c. filtrate in arm, 10 c.c. in bladder
1-25	0		3 c.c. filtrate in arm, 10 c.c. in bladder
2-15	0	++	Gave three doses to be given at home
4-4	0	0	

It will be seen that the bacterial count has continued to be decreased. The day following the first combined treatment the urine became sterile. Because of the difficulty in securing the child's cooperation catheterization had to be abandoned. On February 15, 1930 we succeeded in securing a specimen. It showed ++ B. coli. Three doses of bacteriophage were given to be administered at home. On April 4, 1930 the urine was sterile and the patient had been free from symptoms since January. Even though there was little improvement noted following the first series of subcutaneous injections the parents, unusually intelligent people, say she was always improved in a general way. This was more marked following the second series. She was very much less irritable.

CASE 34.—Caroline P., aged twenty. C. H. The patient has been under observation since 1914, when she was five years old. At this time she gave a history of febrile attacks of seven to ten days' duration, coming on periodically since early infancy. The fever ranged between 101° and 103°. The attacks stopped spontaneously and recurred at intervals of three to seven months. One attack was accompanied by severe chills and cyanosis. Examination revealed an enlarged left kidney, pyuria with large numbers of mononuclear

cells (lymphocytes). In the absence of tubercle bacilli this finding led to the diagnosis of tuberculosis of the kidney, which was proved a year or two later by finding tubercle bacilli and by nephrectomy. She was studied again in 1925; albuminuria was found. There was no increase in cells. Guinea pig inoculation was negative for tuberculosis. In 1926 and 1927 albuminuria was + and ++ with only slight excess of cells. In 1929 albuminuria was still present and many B. coli. There were no symptoms referable to the urinary tract. An entirely satisfactory bacteriophage was not obtained after a long effort. We decided to try it and continued to enhance its virulence. The urine finally became sterile two days after we secured an entirely satisfactory filtrate. The patient had a very severe reaction following the injection on January 22.

CASE 34

DATE	CELLS	B. COLI	REMARKS
1929			
6-5	++	++++	Alkalinization begun. First series
7-23			3 c.c. bacteriophage subcutaneously
7-25	0	++++	3 c.c. bacteriophage subcutaneously
7-27			3 c.c. bacteriophage subcutaneously
7-29		++++	No improvement in bacilluria
9-17	0		3 c.c. bacteriophage subcutaneously. Second series
9-19			3 c.c. bacteriophage subcutaneously
9-21			3 c.c. bacteriophage subcutaneously
9-23	0	++++	No improvement in bacilluria
1930			
1-17	0	++++	
1-18	0		3 c.c. bacteriophage subcutaneously, 10 c.c. in bladder Third series
1-20	0	0	3 c.c. bacteriophage subcutaneously, 10 c.c. in bladder
1-22	0	0	3 c.c. bacteriophage subcutaneously, 10 c.c. in bladder
4-9	0	0	3 c.c. bacteriophage subcutaneously, 10 c.c. in bladder

CASE 35.—A. B. C., aged fifty. C. H. June 6, 1929. Patient came in with a history of pyuria dating from an attack of typhoid fever in 1895 (thirty-four years ago) which was complicated by a severe cystitis. Pus has not completely disappeared from the urine since this time. In 1896 kidney stones were removed. The operation was not entirely successful and a second operation was done in 1898. The pyuria has been more marked since this time. If the patient is careful to drink large quantities of water he gets along very well, but the bad odor continues and is noticeable at all times in his bathroom. He has no other complaints. He looks well and feels well. He is anxious to have the urinary infection cleared up, particularly the bad odor.

Since his operations he has been up at night two or three times to urinate. About once a year he has a digestive upset which is characterized by nausea, vomiting, and high fever. This sends him to bed for a week. There are no urinary symptoms at these times. However, if he does not persist in drinking comparatively large amounts of water there will be irritation in the bladder and bad odor to the urine. Colon bacilli were found in the urine by a competent observer in 1899. The last time they were looked for, eight or ten years ago, they were still present.

Past Treatment: In addition to a large water intake, urotropin has been taken more or less systematically for many years. It finally had to be discontinued because it "irritated the stomach."

Physical Examination: Patient was well nourished and well muscled. The tonsils were in; no history of infection. Teeth were negative. Joints were negative. The lungs and heart were negative. Blood pressure 148/90. Abdomen negative. Appendix scar—1927. Genitals and prostate negative. Slight pruritis ani.

Urine: Turbid yellow, acid, 1.020, odor of infection. No albumin. No sugar. The sediment showed many motile bacilli. Several samples gave the same findings. The gravity varied from 1.009 to 1.019 on the Mosenthal test. The night output was slightly greater than the day.

Bacteriology: A catheterized specimen showed four distinct strains or types of B. coli. Colonies developed on Endos plates as follows:

No. 1. Large luxuriant flat colonies 2 mm. in diameter with red metallic scum, visible in twenty-four hours.

No. 2. Medium sized colonies 1 mm. in diameter with red metallic scum in twenty-four hours.

No. 3. Small colonies with mucous surface. No metallic scum in twenty-four hours. Mucous surface and red metallic scum in forty-eight hours.

No. 4. Tiny colonies showing faint scum with hand lens in seventy-two hours.

Tiny colonies showing white in nine-six hours.

Tiny colonies showing pink in one hundred twenty hours.

Tiny colonies showing red metallic luster in one hundred forty-four hours.

When subcultured on Endos medium, these colony types always develop in the same manner. Transplants of Type 1 remained the same for over seven days. After four months transplants behaved in the same manner. Types 2 and 3 showed no change with the same technic. Type 4 after alternately transplanting in broth and on hard media showed no change in its colony characteristics. One specimen on continuous incubation without opening the Petri dish for five days showed two typical large Type 1 colonies. The following morning, the sixth day, two more Type 1 colonies had developed. These large colonies possessed all the characteristics of Type 1. When transplanted, they developed in twenty-four hours and showed a red metallic scum. We have never been able to produce small or tiny colonies from Type 1 colony. These colony types always run true to form.

On three occasions we have transplanted tiny, Type 4 colonies into broth for twenty-four hours, then back onto Endos plates, resulting in nearly a pure culture of Type 1 which covered the plates. On one occasion it was five days before large colonies appeared, on two occasions it was three days before large colonies appeared on Endos.

On blood agar all colony types become distinct white colonies in twenty-four hours. Type 4 is barely visible to the naked eye (0.5 mm.). It attains its normal size, 1 mm., in diameter, only after forty-eight hours.

In broth a faint haze is produced with Types 1, 2 and 3 in twenty-four hours. It is difficult to carry Type 4 through a broth transplant without change in form, even though the transplants are made every nine hours. Thus it will be seen that this type, which is the most difficult to irradicate from the patient's urinary tract, readily develops into Type 1 under proper cultural conditions.

Action on Sugars. Types 1, 2, 3, and 4 ferment dextrose and lactose. They do not ferment saccharose.

Developing the Bacteriophage.—Sewage base was inoculated with the patient's organisms on June 6, 1929. Feedings and filtrations were done every 12 hours for 35 days before any favorable lysis was produced, and then only on Types 1, 2 and 3 (July 11, 1929). Feedings and filtrations were continued until September 4 before a phage was obtained that was active for Type 4. As will be seen from the treatment chart, it was not difficult to develop a bacteriophage for the first three types with the exception of our first effort. It has been more difficult to secure a filtrate lytic for Type 4. None of our stock filtrates were effective.

Treatment of case with phage filtrate.—As previously pointed out, bringing the P_H of the patient's urine to the neutral or slightly alkaline point is an essential feature in the treatment of colon infections of the urinary tract with Coli-phage. We were fortunate in having a patient who could give us perfect cooperation. It has been necessary to omit treatments for long periods of time on several occasions. This has given us opportunity to investigate the permanency of bacteriophage sterilization.

Subcutaneous Injection.—As a matter of interest a dose of filtrate was given subcutaneously June 14. It had not been developed to a satisfactory point at this time. Cowie has observed that occasionally filtrates that are not completely lytic in vitro may affect the organism when given subcutaneously. There was no such action in this instance. However, twenty-four hours after the second dose Types 1, 2, and 3 disappeared from the urine

and have continued to be absent, with the exception of one occasion, for a year and nine months (April 7, 1930). Type 4 was definitely affected twenty-four hours after the third dose of filtrate (July 16), but was as resistant as ever eight days later despite the fact that two additional treatments had been given and the filtrate was being enhanced daily during this time. On September 4 the first bacteriophage active for Type 4 was obtained. The first negative Type 4 culture was obtained December 2, 144 days after the first of fourteen 3 c.c. subcutaneous injections. The urine remained free from Type 4 for five days, perhaps longer, but showed a ++ growth on the eighth day (December 10), a + growth on the twenty-fifth day, a negative growth on the thirty-first day. The urine was found completely sterile to all types for fifteen days thereafter. The patient went to Florida, returning thirty-five days later. At this time, he was symptom free, the urine was fairly clear, but it gave a +++ growth of Type 4 colonies. This type persisted for twenty-seven days and became negative after the eighth treatment.

Bladder Instillation.—It was our purpose to gain as much information as possible on the effect of the filtrate by subcutaneous injection. Bladder instillations were not added until December 27, 1929. After the bladder was emptied, without bladder irrigation, 10 c.c. of filtrate were instilled. The patient was asked to hold his urine as long as he could comfortably. At first he remained in bed, but after a few trials he was able to do as well by being about. He could hold the filtrate from four to six hours. There has been greater improvement since this time, but the reader will decide for himself by reference to the treatment chart whether this is due in greater part to the bladder instillation or to persistence in treatment. Our feeling, from work on other cases, is that the double method is the better one.

Cells.—The patient's urine at the beginning of observation was definitely cloudy. The uncentrifuged specimen showed clumps and an excess of leucocytes which we marked as ++++. The urine remained in this condition until after the third subcutaneous injection of bacteriophage filtrate. Seldom since this time has there been any appreciable cloudiness. On a few occasions the morning urine, following a bladder instillation the evening before, has been cloudy because of an amorphous detritus. On such occasions the urine was found to be sterile.

Odor.—There had been an unpleasant odor to the patient's urine since his kidney operations in 1896 and 1898. As previously stated this was marked enough to produce a characteristic odor to the bathroom. The odor could be lessened by drinking freely of water. By reference to the treatment table it will be seen that the odor became much less after the third subcutaneous injection of bacteriophage filtrate. It had completely disappeared November 26, 1929, one hundred four days after the first treatment (12 treatments in all, 36 c.c. of filtrate). At this time the patient volunteered the information: "That is the first time my urine has been free from odor in 30 years." It was actually thirty-three years. The odor has not returned. The urine for the most part has been clear as a normal urine—influences aside from pyuria at times have imparted a cloudiness.

Treatment.—The patient has taken soda constantly since beginning treatment. The total period covers about two years. There may have been a few days at a time when soda was omitted. Three level teaspoonsful a day has been the dose. Oranges and grapefruit have been taken freely, and during the past year milk sugar. This regimen has often resulted in a urine alkaline to litmus, more often neutral. There have been no symptoms attributable to the soda. There has never been any edema.

Bacteriophage Filtrate.—A great effort has been made to secure filtrates as free as possible from color and protein. Redness, heat, and swelling frequently occurred at the site of injection, more often when the color was definite and when the dilution of the broth was not as great as it should be. The best filtrates were those developed with synthetic media, and those that could be developed to a high phage titer and which could consequently be more highly diluted. By reference to the treatment table it will be seen that in the first period of observation, June 6, 1929 to January 18, 1930, two hundred twenty-five days or seven and one-half months, we were successful in promptly overcoming B. coli Types 1, 2 and 3. Three doses of filtrate of 3 c.c. each accomplished this. Type 4 disappeared De-

cember 2, eighty-six days after the first of 5 treatments with a potent bacteriophage. The urine remained practically sterile to all types for forty-seven days when this period of observation ended. There was now an interval of one month. The second period of observation continued for over six months, from February 22 to September 4, 1930. At the beginning of this period the first three types were continuously absent from the catheterized specimen. There was, however, a +++ growth of Type 4. After 8 subcutaneous injections and bladder instillations the urine became sterile, but from time to time Type 4 appeared as will be seen by consulting the chart. An interval of four months elapsed before another observation was made. The urine was sterile to all types. There followed an interval of two months without treatment at the end of which Type 4 was ++++. It responded well to treatment but it frequently reappeared in the urine.

The third period of observation covered ninety-eight days, from May 29, 1930 to September 4, 1930. At the beginning of this period the urine was still clear and odorless. On direct examination a few motile bacilli were observed. A combined treatment with the previous filtrate which had been developed further was given. The patient could not return for ten days. At this time the urine continued to be negative to Types 1, 2, and 3, and showed a + culture of Type 4. A week later the urine was again negative for all four types and remained so for at least five days, showing only a few organisms on the eleventh day, increasing to +++ on the twenty-fifth day in spite of three combined treatments.

It will be seen that during the course of these observations one might have been satisfied that the patient was cured had he been discharged January 18, 1930 or particularly had he been discharged November 7, 1930, when two urine samples were negative to all four types four months after the last treatment.

CASE 35

DATE	CLEAR OR CLOUDY	ODOR	CELLS	DIRECT BACT.	CULTURE TYPES 1	2	3	4	CLINICAL NOTES
1929									
6-6	Cloudy	Foul	++++	++++	+++	+++	+++	++++	Alkalinization begun
6-12	Cloudy	Foul	++++	++++	+++	+++	+++	++++	Alkalinization begun
6-13	Cloudy	Foul	++++	++++	+++	+++	+++	++++	Alkalinization begun
6-14	Cloudy	Foul							3 c.c. in arm. Stock bacteriophage
6-15	Cloudy	Foul	++++	++++	+++	+++	+++	++++	
7-11	Cloudy	Foul	++++	++++	+++	+++	+++	++++	3 c.c. in arm. Filtrate lytic for 1, 2 and 3
7-13	Cloudy	Foul	++++	++++	+++	+++	+++	++++	3 c.c. in arm
7-15	Clear	Foul	0	+++	0	0	0	++++	3 c.c. in arm
7-16					0	0	0	+	3 c.c. in arm. 18 hr. after third phage
7-19	Clear	Sl.*							3 c.c. in arm
7-22	Clear	Sl.							3 c.c. in arm
7-24	Clear	Sl.	0		0	0	0	++++	3 c.c. in arm
7-25	Clear	Sl.			0	0	0	+	
7-30	Clear	Sl.			0	0	0	++++	3 c.c. in arm
8-6	Clear	Sl.			0	0	0	++++	3 c.c. in arm
9-4	Clear	Sl.	0		0	0	0	++++	3 c.c. in arm. First phage found active for 4
9-6	Clear	Sl.							3 c.c. in arm
9-9	Clear	Sl.							3 c.c. in arm
9-11	Clear	Sl.	0		0	0	0	++	
9-25	Clear	Sl.			0	0	0	±	
10-18	Clear	Sl.	++	++					
11-26	Clear	0	±		0	0	0	++++	3 c.c. in arm
11-29	Clear	0							3 c.c. in arm. Urine unusually clear. "First time free from odor in 30 years"
12-2	Clear	0	0		0	0	0	0	
12-6					0	0	0	0	
12-7					0	0	0	0	

*Slightly foul.

CASE 35 (Cont'd)

DATE	CLEAR OR CLOUDY	ODOR	CELLS	DIRECT BACT.	CULTURE TYPES 1	2	3	4	CLINICAL NOTES
1929									
12-10	Clear	0	++					++	3 c.c. in arm
12-27	Clear	0			0	0	0	+	3 c.c. in arm, 10 c.c. in bladder
1930									
1-3	Clear	0	0		0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
1-4	Clear	0			0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
1-6		0			0	0	0	0	
1-10		0			0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
1-11	Cloudy	0	0	0	0	0	0	0	Amorphous detritus. Held filtrate all night
1-17	Clear	0	0	0					3 c.c. in arm, 10 c.c. in bladder
1-18	Clear	0	0	0	0	0	0	0	Went to Florida
			Interval of One Month Since Last Treatment						
2-22	Clear	0	+	+	0	0	0	+++	3 c.c. in arm, 10 c.c. in bladder. Returned from Florida
2-24	Clear	0	0	0	0	0	0	++	3 c.c. in arm, 10 c.c. in bladder. 48 hr. after phage
2-28	Clear		++	++	0	0	0	+++	3 c.c. in arm, 10 c.c. in bladder. Before treatment
3-4	Clear	0	0	0	0	0	0	+++	3 c.c. in arm, 10 c.c. in bladder. Before treatment
3-5	Clear	0	0	0	0	0	0	+++	18 hr. after last phage
3-7	Cloudy	0	+	0	0	0	0	++	3 c.c. in arm, 10 c.c. in bladder. Before treatment
3-8	Cloudy	0		0	0	0	0	++	15 hr. after last phage
3-10	Cloudy	0	0	+	0	0	0	++	3 c.c. in arm, 10 c.c. in bladder. Before treatment
3-11	Cloudy	0	0	+	0	0	0	++	15 hr. after last phage
3-14	Cloudy	0	0	0	0	0	0	++	3 c.c. in arm, 10 c.c. in bladder. Before treatment
3-21	Cloudy	0	+	++	0	0	0	±	3 c.c. in arm, 10 c.c. in bladder. Before treatment
3-27	Cloudy	0	0	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder. Before treatment
4-7	Clear	0	0	0	++			++	3 c.c. in arm, 10 c.c. in bladder
4-11	Clear							±	3 c.c. in arm, 10 c.c. in bladder
4-18	Clear	0	+	++					
5-29	Clear	0	±	+					3 c.c. in arm, 10 c.c. in bladder. After 46-day absence
6-9	Clear	0	+	0	0	0	0	+	Filtrate active for Type 4 of Dec. 27, 1929
6-16	Clear	0	+	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
6-20	Clear	0			0	0	0	0	Cultures of sixteenth still negative
6-21	Clear	0	+	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
6-27	Clear	0	++	0	0	0	0	±	3 c.c. in arm, 10 c.c. in bladder
7-3	Clear	0	±	0	0	0	0	+	3 c.c. in arm, 10 c.c. in bladder
7-11	Clear	0	0	0	0	0	0	+++	3 c.c. in arm, 10 c.c. in bladder
9-4	Clear	0			0	0	0	+++	2 months since last treatment
			Interval of Four Months Since Last Treatment						
11-7	Clear	0	0	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder. 4 months since last treatment
11-8	Clear	0	0	0	0	0	0	0	16 hours after phage
11-13	Clear	0	+	+	0	0	0	++	
			Interval of Two Months Since Last Treatment						
1931									
1-9	Clear	0	++	++	0	0	0	++++	3 c.c. in arm, 10 c.c. in bladder
1-10	Clear	0	0	0	0	0	0	0	
1-18	Clear	0	0	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
1-19	Clear	0	0	0	0	0	0	0	
1-23	Clear	0	0	0	0	0	0	±	3 c.c. in arm, 10 c.c. in bladder
1-24	Clear	0	0	0	0	0	0	0	

CASE 35 (Cont'd)

DATE	CLEAR OR CLOUDY	ODOR	CELLS	DIRECT BACT.	CULTURE TYPES 1	2	3	4	CLINICAL NOTES
1931									
1-30	Clear	0	0	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
1-31	Clear	0	0	0	0	0	0	±	
2-6	Clear	0	0	0	0	0	0	++	3 c.c. in arm, 10 c.c. in bladder
2-7	Clear	0	0	0	0	0	0	±	
2-12									3 c.c. in arm, 10 c.c. in bladder
2-20	Clear	0	0	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder. Urine acid
2-21	Clear	0	0	0	0	0	0	0	
2-25	Clear	0	0	0	0	0	0	0	3 c.c. in arm, 10 c.c. in bladder
2-26	Clear	0	0	0	0	0	0	0	
3-6			±					++	
3-7								±	
3-20				0	0	0	0	0	
3-21				0	0	0	0	0	Urine alkaline
3-25				0	0	0	0	0	
3-26				0	0	0	0	0	73 observations to date
1932									
1-6	Patient reports he is still symptom free and urine is odorless**								

**1-27-32 after going to press. Urine sample showed ++++ type A B. coli. No symptoms.

CASE 36.—Ada S., aged two. U. H. 220180. June 29, 1929. Patient brought to Hospital because of cleft palate when she was nine months old. Had influenza at three months and frequent upper respiratory infections. No complaints at present. Examination aside from harelip negative.

February 3, 1930. Returned for cleft palate. During last two weeks has had a temperature as high as 104°. Pneumonia was suspected. Has some cough and some nasal discharge. Examination negative except for evidence of undernutrition and the congenital defect.

CASE 36

DATE	REACTION	CELLS	B. COLI	CLINICAL NOTES
1930				
2-5		++++	++++	Alkalinization begun
2-11	alk.	++++		
2-19		++		
2-27			++++	
3-11		++	++++	2 c.c. filtrate subcutaneously } Given without
3-19		++		1 c.c. filtrate subcutaneously } our knowledge
3-21				Discharged
3-25				Returned. Paracentesis right ear
3-31		++		Alkali discontinued
4-15		+	++++	
4-16		++		Cystoscoped. Dilatation left kidney pelvis
4-25				Cystoscoped. Dilatation right kidney pelvis
11-29		±	++++	Readmitted
12-3	acid			Alkalinization begun
12-12	acid			Increased alkali
12-26				Developed chickenpox
1-7		++	++++	Returned from Contagious Ward
1-14		++	++++	3 c.c. filtrate in arm, 7 c.c. in bladder
1-16			++++	3 c.c. filtrate in arm, 7 c.c. in bladder
1-18			++++	
3-17	acid	++	++++	3 c.c. filtrate in arm, 7 c.c. in bladder
4-1				Discharged. We have not been able on constant effort to develop a filtrate completely lytic for patient's organism

February 5, 1930. Urine contained "pus." Catheterized specimen B. coli ++++. We were unsuccessful in developing a bacteriophage that would satisfactorily lyse the organism isolated from this patient's urine.

CASE 37.—Ida L., aged eighteen months. U. H. 222282. July 29, 1929. Patient brought to the Hospital because of an unexplained fever. Two months ago fever, vomiting, loose stools and anorexia developed. Since this time she has had an afternoon elevation of temperature ranging between 102° and 105°. She has had night sweats during the past week. The mother thinks the patient has pain when she attempts to urinate.

Examination: Aside from a slight general adenopathy the physical examination was negative. Urine: acid, 50 to 70 leucocytes per low power uncentrifuged field, no casts, no blood, albumin negative. There was a definite secondary anemia. Hemoglobin 45 per cent Sahli. Leucocytes 14,000. Kahn negative. Alkalinization was begun. The effect of bacteriophage treatment in this case was very satisfactory. A suitable bacteriophage filtrate was developed in eight days.

CASE 37

DATE	CELLS	B. COLI	CLINICAL NOTES
1929			
7-27	+++	++++	
8-7	++	++++	Alkalinization begun July 29, 1931
8-14	++	++++	Hemoglobin 63 per cent. Leucocytes 19,200
8-16	++	++++	2 c.c. filtrate subcutaneously in arm
8-17		++++	
8-19			2 c.c. filtrate subcutaneously in arm
8-20	0	+++	
8-21			2 c.c. filtrate subcutaneously in arm
8-22	0	+	
8-24	0	±	
8-26	0	±	Hemoglobin 65 per cent
8-28	0	0	
8-30	0	±	1 c.c. filtrate subcutaneously in arm
8-31	0	±	
9-4	0	±	
9-6	0	±	3 c.c. filtrate subcutaneously in arm
9-7	0	±	
9-9			25 c.c. whole blood intramuscularly
9-11			1 c.c. filtrate subcutaneously in arm
9-12		±	
9-26		±	25 c.c. whole blood intramuscularly
10-1			Discharged on sodium bicarbonate. Return in 1 month
10-5	0	0	
10-6	0	0	
10-7	0	0	

CASE 38.—Mrs. D., aged fifty. C. H. September 14, 1929. Patient operated upon for a small hemorrhoid and fissure in ano. Ten days later she developed bladder and urethral irritation. The urine showed no pus but on culture contained ++++ B. coli. There was a history of similar attacks over a period of two or more years. The bacteriophage findings are those of a long standing infection. This patient recovered symptomatically after the fifth treatment but we were unable to sterilize the urine at any time. A recent letter (December, 1931) from this patient reports she is symptom free.

CASE 39.—Lois S., aged seven. C. H. October 28, 1929. The middle of June the patient contracted a "cold" which did not respond to treatment. Since this time there have been frequent recurring temperature increases up to 100° F. During the past three months she has been confined to bed because of the suspicion of tuberculosis. Recently the increased temperature has occurred every day, and she has complained of "spasmodic, shooting, abdominal pain from the navel down." Sometimes the pain may last for half an hour. It may be initiated by an evacuation of the bowels. Her weight has remained stationary at

CASE 38

DATE	CELLS	B. COLI	CLINICAL NOTES
1929			
9-24	±	++++	
10-12		+++	
10-16			3 c.c. filtrate in arm
10-17	+++	++++	
10-18			3 c.c. filtrate in arm
10-21			3 c.c. filtrate in arm
10-22	+++	++	
10-23			3 c.c. filtrate in arm
10-24	+	++	
10-30		±	
10-31			3 c.c. filtrate in arm
11-1	0	±	
11-3			3 c.c. filtrate in arm
11-5			3 c.c. filtrate in arm
11-6		++	
11-8		+	
11-11		+	
11-12		++	3 c.c. filtrate in arm
11-13		++	
1930			
4-28		+++	Large colony type. No symptoms

51 pounds. Sweating occurred for a short period in the beginning of the illness. Recently urinary frequency and burning micturition developed.

Physical examination was negative except for a marked accumulation of smegma and considerable irritation of the external genitals. X-ray examination of the lungs was negative. A catheterized urine specimen showed no excess of cells, no casts, no abnormal chemistry. At the end of twenty-four hours on blood agar and Endos medium the culture was negative. At the end of thirty-six hours in brain heart infusion a positive growth of B. coli was secured. Alkalinization was begun. It took many days (25) to adapt a bacteriophage for this organism. Treatment was begun November 27, 1929.

CASE 39

DATE	CELLS	B. COLI	REMARKS
1929			
10-28	0	+	In brain heart infusion
10-30		±	
11-27	0	+	3 c.c. filtrate in arm
11-29	0	±	3 c.c. filtrate in arm
12-1			3 c.c. filtrate in arm
12-2	0	0	Culture still negative after 5 days
12-16	0	0	3 c.c. of filtrate in arm
12-17	0	0	18 hours after last treatment

The patient has been very much better. Occasionally a slight temperature increase is found. She has been having a mild upper respiratory infection that may be responsible.

April 2, 1930. Patient still continues to run an occasional temperature of 100° F. She looks well, but has gained only half a pound. There are no urinary symptoms or signs. Culture negative. This is a case of previously unexplained low fever associated with a resistant B. Coli type of infection of the genitourinary tract which responded quite promptly to treatment after a prolonged effort to secure a satisfactory bacteriophage.

Bacteriologic Note.—October 28, 1929. Endos and blood agar plates negative in twenty-four hours. Brain heart infusion positive in thirty-six hours. Transplants to Endos and blood agar positive in twenty-four hours, 14 colonies per standard loop. These

were medium sized type R. B. coli colonies. Hemolytic, translucent colonies on blood agar, metallic luster colonies on Endos.

CASE 40.—Mrs. S., aged thirty-five. C. H. October 29, 1929. Patient has had a colon bacillus infection of the urinary tract for many months. Aside from a general indisposition and occasional local urinary irritation, the condition has not been marked. It is desired to have the infection cleared up, as it may be a contributing factor to her feeling of indisposition. This patient responded quickly to bacteriophage treatment.

CASE 40

DATE	CELLS	B. COLI	REMARKS
1929			
10-29	0	+	Alkalinization begun
11-27	0	+	3 c.c. filtrate in arm
11-29	0	±	3 c.c. filtrate in arm
12-2		0	3 c.c. filtrate in arm
12-16	0	0	3 c.c. filtrate in arm
12-18			1.5 c.c. filtrate in arm
12-19	0	0	2 c.c. filtrate in arm
12-20	0	0	
1930			
4-2	0	0	10 c.c. filtrate in bladder

April 2, 1930. The urinary symptoms had been very much improved until two weeks ago when there was a recurrence. "Rheumatic" symptoms developed in the right loin at this time and continued. On general principles 10 c.c. of the bacteriophage filtrate were injected into the bladder. While doing this many small polypoid growths were seen in and around the meatus urinarius and on either side of the vagina posteriorly. Culture of the urine was negative. The symptoms were coming from the polypoid growths.

CASE 41.—Pearl K., aged ten. U. H. 231448. January 5, 1930. Patient has been on Bone and Joint Service for twenty-six days because of poliomyelitis residual palsies. Developed scarlet fever today.

CASE 41

DATE	CELLS	B. COLI	CLINICAL FINDINGS
1930			
1-14	++++	++++	Alkalinization begun. Temperature 103°
1-16	++	++++	Running a septic temperature; severe epistaxis
1-19			Measles developing
1-20			Rash well out
1-21			Rash has subsided. General condition improved
1-22			Unable to alkalinize urine. Increase bicarbonate
			Pain right ear; spontaneous rupture
1-25			Right ear drum perforated. Urine alkaline. Marked cervical adenitis
1-26			Pain over left frontal and maxillary sinuses. Left eyelids swollen
1-28			Colicky pains upper left abdomen. General condition improved
1-30			Pain left ear. Temperature normal
2-3			Fifth day of normal temperature
2-5	++	++++	Seventh day of normal temperature
2-8			Transferred from Contagious ward to Bone and Joint ward
2-20	++++	++++	Referred to Pediatrics for treatment
2-22	++++	++++	3 c.c. filtrate subcutaneously
2-23	0	++++	
2-24	0	+	
2-26	0	+	3 c.c. filtrate subcutaneously, 5 c.c. in bladder
2-27	0	+++	5 c.c. filtrate in bladder
3-17		++	Case became lost to our service after this time
8-5	++++	+++	Returns for observation after three months' absence from Hospital Has been well. Examination reveals septic tonsils, urine pus. No albumin or casts. Culture shows many B. coli

January 12, 1930. Symptoms of scarlet fever have subsided.

January 14, 1930. A fever of 103° developed today. General examination negative. Urine shows pus, no casts, a large number of gram-negative bacilli. Alkalinization was begun. Seven P.M. temperature 104.8°. Face flushed. This patient was not properly cared for. Being on another service she was inadvertently overlooked. There was a definite clearing up of the pyuria and a modification in the number of bacilli following treatment but this was not permanent.

CASE 42.—Louise H., aged two. U. H. 246377. August 9, 1930. Patient brought to Hospital because of undernutrition. Scarlet fever developed May 17, acute throat June 19. Throat was lanced. Infected ears opened several times. In Hospital four weeks. Became rapidly emaciated after this.

Examination: Emaciated, dehydrated, slight rigidity of neck. Leucocytes 19,600, 82 per cent polys, hemoglobin 70 per cent. Spinal fluid clear, under slightly increased pressure. Fundi negative. Guinea pig inoculated, later negative. Blood culture negative. Transferred to Neurosurgery because of suspicion of brain abscess. Ventricular puncture negative.

August 22, 1930. Patient transferred back to Pediatrics.

September 8, 1930. Improving. Has gained 2½ pounds.

September 11, 1930. Fever for past two days. Urine contains pus and bacilli. Culture started. Alkalinization begun. This patient made a satisfactory recovery.

CASE 42

DATE	CELLS	B. COLI	CLINICAL NOTES
1930			
9-11	++++	+++	Alkalinization begun. Fever
9-19	±		Urine is almost clear. Temperature normal all day
9-25	0		Much improved. Leucocytes 14,000. Temp. 100°. Sitting up
10-1	+++		Measles. Transferred to Contagious Hospital
10-14			Returned to ward
10-19	++++		Fever
10-24			Bacteriophage given } Dose not recorded.
10-27			Bacteriophage given } Probably 3 c.c. in arm,
10-28			Bacteriophage given } 7 c.c. in bladder
11-2		++	
11-21	0	±	
11-26	0	0	
12-3			Discharged
1931			
1-4	0	0	Returned for check in the Out Patient Department
			Direct Smear—
			Advised to return in one month. Patient had just recovered from chickenpox

CASE 43.—Euphemia R., aged seven. U. H. 253825. November 21, 1930. Patient brought to Hospital because of attacks of "indigestion" which have been present at intervals for the past two years, irregular cramplike pains over the lower abdomen, nausea, occasional vomiting of food eaten at the previous meal, fever. The urine is said to have contained "pus." Her physician sends her to the Hospital for bacteriophage treatment.

Examination.—Well nourished child. Temperature 100°. Submaxillary and axillary glands palpable. Otherwise a carefully done physical examination was negative. The abdomen showed no abnormal reactions at any time. Kahn negative. Urine showed 15 to 20 cells per low power uncentrifuged field. Culture showed B. coli ++++. To be observed in Out Patient Clinic. This patient improved symptomatically, but the bacilluria did not clear up.

CASE 43

DATE	CELLS	B. COLI	REACTION	CLINICAL NOTES
1930				
11-21	+	++++	acid	Urine is cloudy. Alkalinization started
12-11	+	++++	alk.	Urine is cloudy
12-17	+	++++	alk.	3 c.c. filtrate in arm, 7 c.c. in bladder. Alk. discontinued*
12-19	0	±	alk.	3 c.c. filtrate in arm, 7 c.c. in bladder
12-21	±	+++	acid	3 c.c. filtrate in arm, 7 c.c. in bladder. Alk. resumed
12-23	0	0	alk.	3 c.c. in arm, 7 c.c. in bladder
12-26	0	0	alk.	Urine has been water clear for several days
1931				
2-26	++	++++	acid	Readmitted. Continues to have occasional attacks and vague lower abdominal pain, every 3 or 4 weeks with frequency. G.I. X-rays negative. Alkalies begun
2-28	+	+++	acid	*Autogenous phage* found in urine
3-1	+	+++		
3-4	+	+++	alk.	
3-5	+	+++	alk.	3 c.c. filtrate in arm, 5 c.c. in bladder
3-7	+	+++	alk.	3 c.c. filtrate in arm, 5 c.c. in bladder
3-9			alk.	3 c.c. filtrate in arm, 5 c.c. in bladder
3-10	±	++	acid	Discharged on alkalies, to report later
4-18				Readmitted. Well since discharge. No pain. Appetite good
4-20	±	+++	alk.	
4-24	±	+++		
4-30				Discharged. Satisfactory filtrate has not been developed
6-8				Readmitted. Has had frequency, enuresis, abdominal pain, distention. Urine said to have "pus" clumps
6-9	±	+++		Autogenous phage negative
7-1				Alkalies begun
7-2		++++		
7-10			alk.	3 c.c. filtrate in arm, 5 c.c. in bladder
7-15	+++	++++	alk.	3 c.c. filtrate in arm, 5 c.c. in bladder
7-17	±	+++	neut.	3 c.c. filtrate in arm, 5 c.c. in bladder
7-19	±	++		
7-21				Discharged. Much improved symptomatically. Continue alkalies. Return for further observation. Patient has not returned (January 10, 1932)

*Alkalinization was begun November 21, 1930. Through a misunderstanding the mother discontinued its use when filtrate injections were begun. This was discovered December 21 when alkalinization was resumed and continued.

CASE 44.—Leola H., aged eight. U. H. 258616. February 12, 1931. Brought to Hospital for "appendicitis." Complained of headache, abdominal pain at first to left of navel, now at McBurney's point, nausea, vomiting. Said to have had an "abscessed kidney" three years ago.

Examination.—Tenderness at McBurney's point, with some rigidity. Left costovertebral tenderness. Leucocytes 10,000. Urine: 10 cells per low power field, an occasional granular cast. Surgery suspects a subsiding appendicitis. Referred to Pediatrics with thought that it may be an upper respiratory infection following a tonsillitis. X-ray of chest and our examination negative.

February 20, 1931. Urine culture B. coli ++++. Few cells.

February 24, 1931. Twelfth day in Hospital. Tenderness over maxillary sinuses. X-ray showed maxillary and ethmoid infection. Otology Department found no definite clinical evidence of sinus disease.

May 15, 1931. Catheterized specimen of urine shows on culture a green producing streptococcus-like organism having no B. coli characteristics excepting that it was easily lysed by B. coli bacteriophage filtrate.

May 19, 1931. The same organism was recovered from patient's urine. It was also easily lysed by Coli phage overnight. When left alone it lysed itself in two days' time. This made it impossible to keep a strain of this interesting organism, as we had previously

CASE 44

DATE	CELLS	B. COLI	REACTION	CLINICAL NOTES
1931				
2-20	+	++++	acid	Alkalinization begun
2-22	±	++++	neut.	
2-23	0	++	acid	*Autophage* found in urine
2-24	±	++++	acid	*Autophage* still present
2-25	±	++++	neut.	Direct smear
	0	0	alk.	Later in day. Culture 0. Active phage present
2-26	++	+++	neut.	
2-27	±	+	alk.	
3-1	±		alk.	
3-3	±	+++	alk.	Autophage again present. Being developed for treatment
3-4	±	+++	alk.	
3-5	±	+++	alk.	3 c.c. filtrate in arm, 7 c.c. in bladder
3-7	±	++	alk.	3 c.c. filtrate in arm, 7 c.c. in bladder
3-9				3 c.c. filtrate in arm, 7 c.c. in bladder
3-10	0	0	neut.	Discharged
4-13	+++			Returns to Out Patient Department. Malaise, maxillary tenderness, septic tonsils
4-14				Discharged on acid treatment. Not referred to laboratory
4-26	+			Returns in good condition. Alkalinization resumed. Sent home
5-15		++++		Returns. Increased frequency, headache, nocturia, malaise. Chill this A.M. Generalized abdominal tenderness. No rigidity. Costovertebral angle tenderness
5-19	++	++++		
6-3		0		*Potent phage found in urine.* Direct smear showed organisms. Disappeared from urine in 24 hours
6-9	±	0		Direct smear. Streptococcus. Reaction of autophage same as June 3. See text May 15, 1931

done with the original strain of B. coli. The lytic principle from this organism, this autolytic principle, was active against the original B. coli isolated from the patient's urine January 20, 1931.

CASE 45.—Mrs. H. L. S., aged fifty. C. H. May 25, 1931. Patient has been having manifestations of pyelitis for many months; chills, urinary frequency, and irritation coming on at intervals. Urotropin and other antiseptics have been used. A catheterized urine specimen showed slight excess of leucocytes, no casts, and on culture B. coli ++++. Alkali-

CASE 45

DATE	CELLS	B. COLI		REMARKS
1931		R	S	
5-25	+	+	+++	Alkalinization begun*
6-19	0	+	0	Urine alkaline
7-6	0	+	0	3 c.c. filtrate in arm, 10 c.c. in bladder
7-7	0	+	0	3 c.c. filtrate in arm, 10 c.c. in bladder
7-8				3 c.c. filtrate in arm, 10 c.c. in bladder
7-9	0	±	0	
7-10	0	++++		3 c.c. filtrate in arm, 10 c.c. in bladder
7-11				Urinary frequency, chills, irritation
7-13	0	±		Symptoms have disappeared
7-14	+	++		
7-20	+	++++		
7-21	0	0		Symptoms still absent
7-28	0	0		Symptoms still absent

*Bacteriology: The first culture showed no growth on Endos medium in twenty-four hours. Numerous very tiny green producing colonies resembling Streptococcus viridans developed on the blood agar plates. When these were transplanted in broth, incubated and again transplanted on Endos, typical B. coli, R. type, colonies developed. The tiny type of colony was never again encountered. It will be observed that alkalinization had been going on for twenty-five days and the urine was alkaline at the time of the second culture.

nization was begun. This patient has remained free from symptoms to date (December 5, 1931).

CASE 46.—Mrs. C. V., aged thirty-one. C. H. September 28, 1931. This patient has had a known colon infection of the urinary tract for over a year. She has previously been treated in New York with a filtrate prepared by us. She was very carefully watched by competent observers and responded fairly well to treatment. The B. coli, however, did not remain away from the urine. At one time the culture is reported to have become negative. She has had no symptoms since this time. She presents herself for examination.

The patient is a very robust person without any symptoms. She comes in for a check-up examination. Aside from the bacilluria the examination was entirely negative. The sequence of events follow.

CASE 46

DATE	CELLS	B. COLI	URINE	REMARKS
1931				
9-28	0	±	acid	Alkalinization begun
9-30	+	++++	acid	
10-13	±	++++	alk.	3 c.c. filtrate in arm, 10 c.c. in bladder
10-14	0	±	alk.	18 hours after first treatment
10-15	0	±	alk.	3 c.c. filtrate in arm, 10 c.c. in bladder
10-16	0	0	alk.	
10-17	0	0	alk.	3 c.c. filtrate in arm, 10 c.c. in bladder
10-19	0	0	neut.	3 c.c. filtrate in arm, 10 c.c. in bladder
10-21	0	+	alk.	3 c.c. new filtrate in arm built from Oct. 17 set-up Fed 3 times a day. It is much more potent
10-22	0	++	alk.	19 hours after last treatment
10-23	0	±	alk.	3 c.c. new filtrate in arm
10-24	0	±	alk.	
10-26	0	++	alk.	3 c.c. new filtrate in arm
10-27	0	+	alk.	
10-29	±	+	alk.	Organisms in the urine are agglutinated. Heretofore they have always been free
10-31	0	±	alk.	
11-2	0	±	neut.	Allowed to leave city for a few days
11-12	±	++++	alk.	Discharged on sodium bicarbonate and basic diet

This has been a very resistant strain of B. coli. It was definitely affected after the first series of injections. We were, however, never able to cause the organism to disappear from the urine for more than a few days. A bacteriophage was not demonstrated in her urine at any time. The patient is perfectly well and has been since her first course of treatment in New York. She was discharged on alkaline treatment with the usual advice.

CASE 47.—Mrs. F. D. G., aged sixty-six. C. H. December 31, 1931. Last summer (August) the patient had symptoms attributed to a cystocele, bearing down sensation interfering with her walking. Replacement and douching overcame the symptoms. A ring pessary was introduced in October. This caused much discomfort in the rectum and had to be abandoned. Another pessary was tried. Three days later bladder symptoms developed; painful micturition and urinary frequency, day and night. This was treated for several days with bladder irrigations. She has taken urotropin since October; but only five grains every morning. Some other urinary antiseptic was used, possibly not more adequately than the urotropin. Recently she has been taking soda. For the past seven years she has taken more or less soda for "gastric ulcer." On entrance urinary frequency and painful urination were still present.

Examination showed a fairly well preserved woman for her years. Glaucoma. Thin edge of liver felt two fingers below the costal margin. No nodules. A few days after the bladder discomfort began she complained of generalized pain in the abdomen and a slight increase in temperature which lasted for five days. Tenderness on pressure over the abdomen at this time; not localized. The perineum is inadequate. There is a rectocele and a cystocele. There is no redness about the urethra. The uterus is normally atrophied.

A catheterized urine specimen showed 150 cells per low power uncentrifuged field with clumps. The culture showed +++ B. coli.

CASE 47

DATE	CELLS	B. COLI	REMARKS
1931			
12-31	+++	+++	Alkalinization continued
1932			
1-6	++	+	Urine alkaline. 3 c.c. bacteriophage in arm, 10 c.c. in bladder
1-8	+	+	Urine alkaline. 3 c.c. bacteriophage in arm, 10 c.c. in bladder
1-11	+	0	Urine alkaline. 3 c.c. bacteriophage in arm, 10 c.c. in bladder
1-13	0	0	Urine alkaline. 3 c.c. bacteriophage in arm, 10 c.c. in bladder

Patient's symptoms have all disappeared.

METHODS USED FOR DEVELOPING BACTERIOPHAGE FOR TREATMENT OF B. COLI INFECTIONS

By the use of our sewage base method* many days' time is saved over other methods of bacteriophage development which we have tried. It is possible to develop a water clear filtrate which gives only a faint pink local reaction at the site of inoculation without the occurrence of edema or a general reaction. Filtrates developed in broth without subsequent development on solid media give marked reactions. Filtrates entirely or finally developed on solid media give only slight reactions. The methods in use at the present time are as follows.

Sewage Base.—Sewer water is filtered clear through gauze, paper and infusorial earth. To 100 c.c. of the resulting filtrate 0.4 gram of dehydrated bactonutrient broth (Difco) is added. After thoroughly mixing, this is sterilized by filtering through a Berkefeld M candle.

Selecting a Bacteriophage.—To twelve sterile test tubes approximately 8 c.c. of the sewage base is added. One of these is used for a control. The others are fed with varying amounts of a nine-hour culture of the clinical strain of B. coli. One tube is filled with broth as a control. Between the ninth to the fifteenth hour these tubes must be frequently and carefully watched, for it has been our observation that the bacteriophage under these conditions does not

TABLE IV

TUBE NO.	CHARACTER OF TUBE	GROWTH RESULT AFTER 9 TO 15 HOURS INCUBATION
1	8 c.c. broth control + 1 mm. loop B. coli suspension	++++
2	8 c.c. filtrate control	0
3	8 c.c. filtrate + 1 mm. loop 9 hr. B. coli suspension	+
4	8 c.c. filtrate	++++
5	8 c.c. filtrate	++
6	8 c.c. filtrate	0
7	8 c.c. filtrate	+++++
8	8 c.c. filtrate	+
9	8 c.c. filtrate	+++
10	8 c.c. filtrate	0
11	8 c.c. filtrate	0
12	8 c.c. filtrate	+

*Cowie, D. M.: Observations on the Bacteriophage, Ann. Clin. Med. **1**: 73, 1926.
Cowie, D. M.: The Present Status of the Bacteriophage in Colon Infections of the K.U.B. tract, Transactions of U. of M. Ped. and Infect. Dis. Society, 1929.

develop in appreciable amount until after nine hours, and that secondary growth of resistant organisms may start as early as the fifteenth hour, or some contaminating organism may render the tubes cloudy, thus obscuring lysis that may have occurred. A set up and results may be illustrated as shown in Table IV.

Building the Stock Phage Filtrate.—Add all the negative and one-plus filtrates together (3, 6, 8, 10, 11, 12). In this instance it will make a batch of 48 c.c. Filter through a Berkefeld M candle to render sterile. It will be seen that each of the tubes selected was able to lyse, to all practical purposes, one loop of the nine-hour broth culture. In other words, the combined filtrates had lysed six loops. It will be safe then to inoculate the combined filtrate with 3 loops of the nine-hour broth culture. After nine hours incubation we usually get a clear fluid.

Feed the resulting fluid with the same amount of culture without filtering if it is clear, otherwise filter and feed. Incubate nine hours as before.

On successive nine-hour feeds gradually increase to 3, 5, 6, 9 loops, and then to 1 c.c. of the culture, repeating each successive increase once. In this way if great care is taken not to feed too much, subsequent filtration may be avoided and much time saved. The point is to feed just enough to prevent visible growth.

This builds our stock or active concentrated bacteriophage filtrate from which we build our individual filtrates for treatment. We keep these stock filtrates constantly developing. Usually nine or ten are always on hand, each one of which may be tested against a patient's strain on agar plates, which shows plainly and most easily whether the filtrate is active for the patient's strain. This information is obtained in from eight to fifteen hours.

Building the Treatment Filtrate.—This may be done in one of three ways.

First Method: Seed one to three plain agar 6-inch plates with two drops of a nine-hour broth growth of the patient's strain. Dry in the incubator for three-quarters of an hour. Cover with two or three drops of the bacteriophage filtrate. Incubate for nine to fifteen hours. Wash off the organisms with about 12 c.c. of sterile saline solution with the aid of a smooth bent glass rod, and pipette into plate No. 2. Wash and in like manner pipette into plate No. 3, using sterile technic throughout. Now pipette the contents of the third plate on to a sterile fourth plain agar plate and incubate all the plates for varying lengths of time, until satisfactory lysis is observed (nine to fifteen hours).

It is generally found that plates 1, 2, and 3 will be covered with growth and plaques, the plaques predominating, while plate 4 will look the same as it did when it was put into the incubator. That is, the added, combined suspension has evaporated very little, and the organisms present have settled to the bottom, giving the appearance of a smooth yellowish white layer on the surface of the agar.

Plates 1, 2, 3, and 4 are now washed a second time with 20 to 30 c.c. of sterile saline solution. The resulting combined mixture is centrifuged until clear. The supernatant fluid is removed, sterilized by passing through a Berkefeld M candle, cultured for sterility and tested for bacteriophage activity by one of the following methods:

(a) By testing the action of varying amounts of filtrate against a nine-hour broth suspension of the clinical strain. This may be done in the following manner. In each of five small, ⅜-inch, test tubes place 10 drops of the broth suspension and serially 1, 2, 3, 4, and 5 drops of the bacteriophage filtrate. The tubes are then put in the incubator and carefully watched between the ninth and twentieth hours. The tubes having the largest amounts of the filtrate are more likely to be found clear. However, we not infrequently find a clear tube among the weaker dilutions. This fact has been demonstrated by D'Herelle.

(b) By adding ¼ c.c. of the filtrate to varying quantities of a nine-hour suspension of the clinical strain, 1, 2, 3, 4, 5, and 6 c.c. respectively. These preparations are incubated and observed in the manner previously described. The tube showing the greatest lyses is accepted as demonstrating the lytic power of the bacteriophage.

(c) By seeding a 3-inch agar plate with 1 to 3 drops of a nine-hour broth suspension of the clinical strain and drying in the incubator for three-quarters of an hour. A square is marked on the bottom of the Petri dish with a wax pencil or a pen. This is the test area. The margins serve as control areas. Spread carefully over the square area one or two drops of the filtrate to be tested and observe in the incubator from the ninth to the twenty-fourth hour. Between these hours it will usually be found that from a few plaques to a complete lysis or a complete inhibition of the growth in the square has been effected, while a luxuriant growth has developed over the control margins of the plate.

Second Method: One hundred to 500 c.c. of the first bacteriophage filtrate are placed in a sterile Erlenmeyer flask. To this is added 1 to 5 c.c. of a nine-hour broth culture of the patient's strain. This mixture is allowed to stand at room temperature for from three to seven days, at which time a heavy growth will be found unless a very potent bacteriophage has developed. This mixture is sterilized by filtration through a Berkefeld M candle. The P_H is corrected to 7.8. The growing factors are again added (0.4 gm. dehydrated bactonutrient broth—Difco) and the fluid is again inoculated with 1 to 5 c.c. of a nine-hour broth suspension of the clinical strain, incubated for three to seven days, and filtered. The P_H is corrected, and this procedure repeated until no further growth results and the fluid remains clear. It is then tested for bacteriophage activity against the clinical strain by the means described under the first method.

Third Method: Take 100 c.c. of the filtrate, add one loop of a broth suspension of the clinical strain, incubate nine hours. The contents of the flask should be clear because purposely only a small feeding has been given. The flask is now fed three times a day. On the first day one drop at each feeding, on the second day three drops, on the third day five drops, on the fourth day ten drops. This develops the potency high enough to test for its therapeutic activity as previously described. The idea of this method is to present to the developing bacteriophage a task it can easily handle in the time allotted; in other words, assuring that the bacteriophage will never be overwhelmed by too large numbers of B. coli. These filtrates may be developed so high that they will stand high dilution, or they may be developed once or twice more on solid media, thus eliminating the great excess of protein.

Comment on the Methods Presented.—The first method eliminates nutrient broth from the final product. The process of washing if carefully done need remove very little of the hard culture media. The degree of dilution seems sufficient to prevent unpleasant reactions in the large percentage of patients. The second method necessarily furnishes a filtrate containing considerable protein, which can be reduced only by diluting the resulting filtrate. Oftentimes the lytic power is great enough to stand diluting sufficiently to overcome undesirable reactions. Great care has to be taken to keep the P_H of this filtrate between 7.4 and 7.6. The filtrate secured by the third method also contains considerable protein. We have felt that by the frequent feeding the lytic principle has often developed more rapidly. For all purposes we think that methods utilizing hard media are preferable because they are less likely to contain substances that may cause reactions and because we are more correct in interpreting beneficial results as being due to the bacteriophage.

CONCLUSIONS

1. Bacteriophage inoculation in colon infections of the urinary passages is an effective method of treatment.

2. Success in treatment with bacteriophage depends upon careful individual adaptation of the bacteriophage corpuscles to the strain or strains of B. coli responsible for the infection, and careful previous preparation of the patient by alkalinization and a continuance of alkalinization for some time after the urine has become sterile.

3. The comfort of the patient following bacteriophage inoculation depends on reducing the protein content of the filtrate to the minimum. Water clear filtrates produce little or no reaction. Colored filtrates almost invariably produce undesirable reactions which seem more likely to occur in adults than in young children.

4. Recent acute B. coli infections are usually quite promptly terminated by bacteriophage inoculation.

5. Chronic B. coli infections are more resistant to the action of the bacteriophage. It is often more difficult to develop a satisfactory filtrate for this type of infection.

6. One course of B. coli bacteriophage inoculations does not prevent subsequent inoculation being effective.

7. Very often long standing infections may be terminated or greatly improved by bacteriophage inoculation.

One is impressed with the feeling that if a bacteriophage can finally be developed that will properly lyse the organism in vitro, sterilization will occur no matter how resistant the strain or how long standing the infection.

9. It appears that no immunity to subsequent attacks is conferred by bacteriophage sterilization of the urinary tract.

The bibliography on the bacteriophage is comprehensively brought up to date in Dr. D'Herelle's books, published by Williams & Wilkins Company. The senior author wishes to express appreciation of the very careful assistance of Mr. Henry Poncher, Mr. Robert Hicks, and Mr. Elmer De Gowin at various times during the progress of these observations.